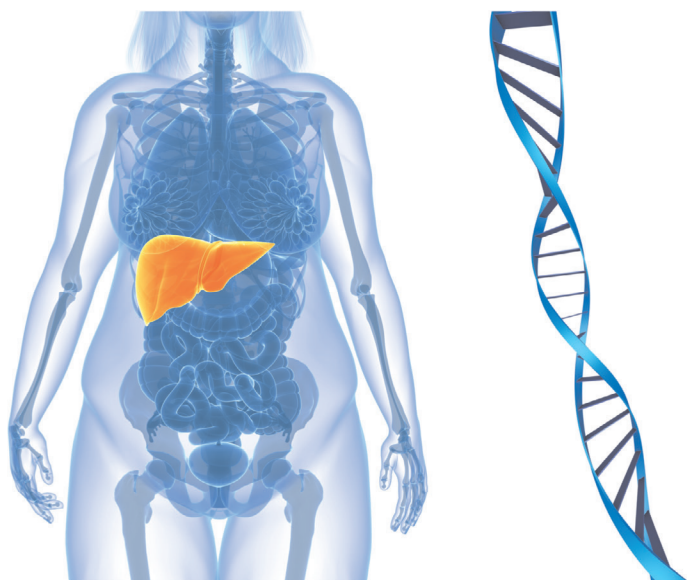


JENNI HYYSALO

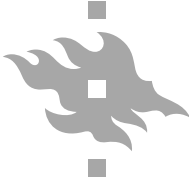
Prevalence and Genetics of Non-Alcoholic Fatty Liver Disease



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PREVALENCE AND GENETICS OF NON-ALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

Introduction: Non-alcoholic fatty liver disease (NAFLD) is a heterogeneous condition and at least two different forms exist. ‘Obese/Metabolic NAFLD’ characterizes subjects with metabolic syndrome and insulin resistance, whereas NAFLD associated with the common *PNPLA3 I148M* variant (‘PNPLA3 NAFLD’) is not accompanied by insulin resistance. The aims of the present studies were to determine 1) whether genetic variation in *APOC3* contributes to liver fat content and plasma triglyceride and apoC3 concentrations, 2) the prevalence of non-alcoholic steatohepatitis in Finnish subjects, and 3) how the *PNPLA3* gene variant influences the serum lipidome.

Subjects and methods: The study groups consisted of 417 (I) and 372 (III) subjects, as well as 296 bariatric surgery patients from Finland and 2849 subjects from a population-based D2D-sample (II). In Study II, 380 non-bariatric surgery patients who had undergone a liver biopsy for suspected NAFLD from Italy were used as an external validation cohort. In Study I liver fat content was measured by proton magnetic resonance spectroscopy (¹H-MRS), in Study II by either biopsy or ¹H-MRS, and in Study III in 75% by ¹H-MRS and in 25% by biopsy. Clinical characteristics (I, II, III), plasma apoC3 concentrations (I), and serum cytokeratin 18 fragments (II) were determined. In Study III, lipidomic analyses were performed using ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-MS). Individuals were genotyped for rs2854116 and rs2854117 in *APOC3* (I) and the known rs738409 in *PNPLA3* (I, II, III). In Study II, we developed and validated a ‘NASH score’ in the Finnish and Italian biopsy cohorts, which was then used to predict NASH prevalence in the population-based D2D study. In Study III, the subjects were divided into groups based on *PNPLA3* genotype or obesity, and the absolute and relative triacylglyceride concentrations were compared between the subgroups.

Results: No difference existed between the *APOC3* variant allele (T-455C or C-482T or both) carriers and non-carriers in liver fat or apoC3 concentrations, whereas those with the *PNPLA3* GG genotype at rs738409 had a 2.7-fold higher liver fat content than those with the CC genotype (I). The ‘NASH score’ included *PNPLA3* genotype, aspartate aminotransferase (AST), and fasting insulin. The area under the ROC for this score was 0.774 (95% CI 0.709-0.839) in Finns and 0.759 (95% CI 0.711-0.807) in Italians (NS) (II). The prevalence of NASH based on this score in the D2D study was 6.0% (95% CI 5.0-6.9%) (II). Sensitivity

analysis was performed by a Bayesian model, which gave a NASH population prevalence of 3.6% (95% CI 0.2-7.7%) using the 'NASH Score' (II). Absolute and relative deficiency of distinct circulating TAGs was observed in the *PNPLA3*^{148MM/148MI} as compared with the *PNPLA3*^{148II} group (III). Genotypes in the obese and 'non-obese' groups were similar but the obese subjects were insulin-resistant (III). Liver fat was increased in both obese and *PNPLA3*^{148MM/148MI} groups (III). Multiple changes in the relative TAG concentrations were observed between obese and 'non-obese' groups (III). These closely resembled those between obese subjects with 'obese NAFLD' versus the 'PNPLA3 NAFLD' (III).

Conclusions: Variation in liver fat cannot be explained by genetic variants in *APOC3* (I). The population-based prevalence of NASH in Finnish subjects is ~5% (II). The circulating TAG profile depends on the aetiology of NAFLD (III). In 'PNPLA3 NAFLD' a relative deficiency of TAGs is observed, supporting the idea that the I148M variant prevents lipolysis rather than stimulates TAG synthesis in the liver (III).

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LIST OF ORIGINAL PUBLICATIONS

I. Hyysalo J, Stojkovic I, Kotronen A, Hakkarainen A, Sevastianova K, Makkonen J, Lundbom N, Rissanen A, Krauss RM, Melander O, Orho-Melander M, Yki-Järvinen H. *Genetic variation in PNPLA3 but not APOC3 influences liver fat in non-alcoholic fatty liver disease*. J Gastroenterol Hepatol. 2012; 27:951-6.

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II. Hyysalo J, Männistö VT, Zhou Y, Arola J, Kärjä V, Leivonen M, Juuti A, Jaser N, Lallukka S, Käkälä P, Venesmaa S, Simonen M, Saltevo J, Moilanen L, Korpi-Hyövalti E, Keinänen-Kiukaanniemi S, Oksa H, Orho-Melander M, Valenti L, Fargion S, Pihlajamäki J, Peltonen M, Yki-Järvinen H. *A population-based study on the prevalence of NASH using scores validated against liver histology*. J Hepatol. 2014; 60:839-46.

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III. Hyysalo J, Gopalacharyulu P, Bian H, Hyötyläinen T, Leivonen M, Jaser N, Juuti A, Honka MJ, Nuutila P, Olkkonen VM, Oresic M, Yki-Järvinen H. *Circulating triacylglycerol signatures in nonalcoholic fatty liver disease associated with the I148M variant in PNPLA3 and with obesity*. Diabetes. 2014; 63:312-22.

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ABBREVIATIONS AND DEFINITIONS

ALA	alpha-linoleic acid
ALD	alcoholic liver disease
ALT	alanine aminotransferase
ANOVA	analysis of variance
Apo	apolipoprotein
ApoC3	apolipoprotein C3 (protein)
APOC3	apolipoprotein C3 (gene)
AST	aspartate aminotransferase
ATGL	adipose tissue triglyceride lipase
AUROC	area under the ROC curve
BMI	body mass index
C	cytosine
Cer	ceramide
CETP	cholesteryl ester transfer protein
CK-18	cytokeratin 18 (M65 antigen)
CLF	chronic liver failure
CM	chylomicron
CRP	C-reactive protein
CT	computer tomography
CVD	cardiovascular disease
DAG	diacylglycerol
DM2	type 2 diabetes
DNL	de novo lipogenesis
ECG	electrocardiogram
EASL	European Association for Study of the Liver
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FA	fatty acid
FAT/CD36	fatty acid translocase/cluster of differentiation 36 (membrane protein)
FATP	fatty acid transport protein
FFA	free fatty acid
FLI	fatty liver index
fP	fasting plasma
fS	fasting serum
G	guanine
GWAS	genome-wide association studies
γ GT	gamma-glutamyltransferase
HBV	hepatitis B
HCC	hepatocellular carcinoma
HCV	hepatitis C
HDL	high-density lipoprotein
HL	hepatic lipase
¹ H-MRS	proton magnetic resonance spectroscopy
HOMA-IR	homeostatic model assessment of insulin resistance
HPLC	high performance liquid chromatography
HSL	hormone-sensitive lipase
HTGC	hepatic triglyceride content

I	isoleucine
IDF	International Diabetes Federation
IL-6	interleukin 6
INS	insulin
IQR	interquartile range
IR	insulin-resistant
IRS-1	insulin receptor substrate 1
LA	linoleic acid
LCAT	lecithin-cholesterol acyl transferase
LDL	low-density lipoprotein
LFAT	liver fat
LPL	lipoprotein lipase
M	methionine
MDB	Mallory-Denk bodies
MG	monoglyceride
MRE	magnetic resonance elastography
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MET	Metabolic Equivalent of Task
MetS	metabolic syndrome
MUFA	monounsaturated fatty acid
NAFL	non-alcoholic fatty liver (simple steatosis)
NAFLD	non-alcoholic fatty liver disease
NAS	NASH activity score
NASH	non-alcoholic steatohepatitis
NICE	National Institute for Health and Care Excellence
NS	not significant
P	plasma
PA	phosphatidic acid
PAI-1	plasminogen activator inhibitor 1
PDFF	proton density fat fraction
PL	phospholipid
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PNPLA3	adiponutrin (gene)
PPAR γ	peroxisome proliferator-activated receptor gamma
PS	Phosphatidylserine
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
S	serum
SAFA	saturated fatty acid
SCD-1	stearoyl CoA desaturase 1
SEM	standard error of mean
SF	serum ferritin
SNP	single nucleotide polymorphism
SREBP-1a	sterol regulatory element binding protein 1a
TAG	triacylglycerol
TCA	tricarboxylic acid cycle

TLR4	toll-like receptor 4
TM6SF2	transmembrane 6 superfamily 2 gene
TNF α	tumour necrosis factor alpha
UPLC-MS	ultra-performance liquid chromatography mass spectrometry
UPR	unfolded protein response
US	ultrasound
VLDL	very low density lipoprotein

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the Western world. NAFLD covers a spectrum of liver disease from simple steatosis to fatty infiltration with inflammation to hepatocellular ballooning degeneration (non-alcoholic steatohepatitis, NASH). The latter may progress to fibrosis and/or cirrhosis, in the absence of excess alcohol consumption (1).

The increase in NAFLD prevalence parallels that of obesity. Insulin resistance and metabolic syndrome develop especially in those obese subjects with fatty liver, termed ‘Obese/Metabolic NAFLD’ in the ensuing discussion (2). In this type of NAFLD, caloric excess and physical inactivity increase hepatic free fatty acid (FFA) influx and hepatic *de novo* lipogenesis (DNL), resulting in excess liver fat (3). This in turn leads to hypertriglyceridemia, hyperglycemia and hyperinsulinemia (3).

A common I148M gene variant in *PNPLA3* has been extensively studied and its robust association with accumulation of liver fat has been independently confirmed in numerous studies (4-9). NAFLD associated with *PNPLA3* rs738409 (‘PNPLA3 NAFLD’) is not per se accompanied by insulin resistance or other metabolic abnormalities, such as hyperinsulinemia and hypertriglyceridemia, as seen in ‘Obese NAFLD’ (3). Other genetic causes for NAFLD, such as variants in *APOCIII* (alleles T-455C and C-482T) have been suggested to exist (10) but have not been confirmed. ‘Obese/Metabolic NAFLD’ and ‘PNPLA3 NAFLD’ may coexist (11). Changes in the distribution of circulating triacylglycerols (TAGs) in the two different types of NAFLD have not been investigated in humans.

The pooled global prevalence of NAFLD is estimated to be around 25% (12). Fatty liver can be diagnosed reliably using imaging techniques, but diagnosis of NASH requires biopsy. Various clinical and biochemical markers have been combined to form diagnostic scores to predict NASH, but they lack diagnostic accuracy. Current biopsy-based prevalence estimates of NASH are from highly selected groups of patients, and no population-based estimates have been published.

REVIEW OF THE LITERATURE

1. Lipid metabolism under physiological conditions

1.1. Overview

Lipids can be divided into various groups based on their chemical structure. In terms of abundance the most prevalent are the triacylglycerides (TAGs) (13). Another important group of lipids that are found in cell membranes and lipoproteins are phospholipids (PLs), which in addition act as precursors for signalling molecules such as diacylglyceride (DAG), phosphatidic acid, eicosanoids, and lyso-phospholipids (14). Fatty acids (FAs) are the elementary units of fats and can be further divided into saturated- (SAFA), mono-unsaturated- (MUFA), and polyunsaturated fatty acids (PUFA). FAs found in the diet contain mostly 16 or 18 carbons. Linolenic acid (LA, 18:2, n-6) and α -linolenic acid (ALA, 18:3, n-3), and long-chain polyunsaturated fatty acids (PUFAs) produced from them, such as eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (DPA, 22:5), and docosahexaenoic acid (DHA 22:6) from ALA, and γ -linolenic acid (GLA, 18:3), dihomogamma-linolenic acid (DGLA, 20:3), and arachidonic acid (AA, 20:4) from LA, cannot be synthesized by the body and are called essential fatty acids (15, 16).

The transport of TAGs and FAs in the circulation differ due to a difference in their hydrophilic nature (17). FAs are transported in the plasma bound to albumin in a free, non-esterified form (free fatty acid, FFA), whereas water-insoluble TAGs are transported in plasma by lipoproteins (14, 18, 19). Lipoprotein lipase (LPL, in muscle and adipose tissue) and the structurally related hepatic lipase (HL, in liver) release FAs from TAGs on the endothelial wall of capillaries (17), after which the FFAs cross the endothelial cell lining down a concentration gradient and/ or via FA transport proteins (FATPs) (20-22).

Fat storage

TAG droplets are concentrated energy stores and are mainly formed by either uptake of FAs from plasma TAGs (in lipoproteins) or by *de novo lipogenesis* (DNL, see below) (23). In a diet rich in dietary fats (*e.g.* Western diet), the more important route by far is uptake of plasma TAGs (24). The most important tissue for storage of fat is the adipose tissue. In the fed, high-insulin state, the adipose tissue LPL activity is enhanced and the freed FAs are taken up by adipocytes via passive diffusion and FAT/CD36 carriers (21, 22). After entering

the cells the FAs can be esterified to form TAGs, a process which is also increased by insulin (25).

Fat mobilization

Fat mobilization from adipose tissue, on the other hand, is intensified by lowering of insulin concentration. Low insulin levels in the fasting state downregulate the adipocyte LPL resulting in lower FA influx (26), whereas increased intracellular hydrolysis leads to increased FA efflux (27). The hydrolysis of TAGs is carried out by the enzymes adipose triglyceride lipase (ATGL, removal of the first FA) and hormone-sensitive lipase (HSL, removal of the second FA) (28), which act on the surface of the TAG droplet. Removal of the third and last FA is carried out by monoacylglycerol lipase (14). The process results in one glycerol and three FA molecules, which mostly leave the cell and enter the circulatory FFA pool. Like LPL, HSL is regulated by insulin and remains active in the fasting state when insulin concentrations are low, and is on the contrary postprandially inactivated by high insulin levels (29).

1.2. Liver lipids

Lipid metabolism in liver includes both oxidation and synthesis of FAs. The main sources of liver FAs include FFAs liberated from adipose tissue, chylomicron remnants, or from the intestine via portal vein and from DNL (24). Upon hepatic uptake, the FAs can be directed to oxidation, ketone body formation, TAG droplets or phospholipid (PL) synthesis, or incorporated into VLDL.

The synthesis of FAs from excess carbohydrates is called *de novo* lipogenesis (23), and although it is relatively small (less than 5% in fasting state) compared with dietary FA intake (18) it plays a key role in coordinating glucose and fat metabolism (30). *De novo* lipogenesis produces exclusively SAFAs such as 16:0 and 18:0. These can then be converted to 16:1 and 18:1 FAs via the action of hepatic stearoyl CoA desaturase 1 (SCD-1) (31).

FAs enter the liver from the circulation as FFAs or from lipoproteins (see below) via lipolysis. When the insulin concentration is low (i.e. in the fasting state), most of the circulating FFAs originate from adipocyte TAG stores via lipolysis. These FFAs released from the adipose tissue account for 80% of the FAs incorporated into VLDL in the liver (18).

Fatty acid oxidation and storage

In the fasting state, when insulin concentrations are low and those of glucagon high, FAs are directed mainly into mitochondrial beta-oxidation to produce energy (32) as well as into VLDL synthesis, instead of storage (18). During the oxidation ketone bodies are produced and released into the circulation.

When insulin concentrations increase in the fed state the FAs are directed into the liver TAG droplets for storage (25), and not into VLDL synthesis (18). This results from the suppression of VLDL assembly, as well as lowering of hepatic FFA uptake by insulin (26).

De novo lipogenesis (DNL)

Another pathway stimulated by insulin and primarily active in the liver is DNL. In this pathway, glucose or other simple sugars or amino acids are taken up by the hepatocyte, and after passing through the glycolytic and tricarboxylic pathway exit the mitochondrion as citrate. Cytoplasmic enzymes catalyse the conversion of citrate into malonyl-CoA (23), which is a key regulator between carbohydrate and fat metabolism (33). The main product of DNL is palmitate, but also stearate and shorter SAFAs are produced.

Under normal conditions, DNL is believed to be a minor contributor to serum TAGs (24). Nevertheless, it has been shown that a diet rich in carbohydrates (especially fructose) increases hepatic DNL contributing to hypertriglyceridemia (34). High carbohydrate intake activates DNL in the liver and increases synthesis of VLDL (35).

1.3. Circulating lipids

Lipids such as FFAs and TAGs are hydrophobic and therefore need carriers for transport in the circulation. FFAs are bound to albumin, whereas TAGs and cholesterol are carried in lipoproteins, which are lipid droplets surrounded by a layer of membrane phospholipids.

Lipoproteins and apolipoproteins

Lipoproteins exist with different lipid and protein compositions, which affect their size. The chylomicrons (CMs) and VLDL are rich in TAGs and larger than LDL and HDL, which are enriched in cholesterol and involved in cholesterol transport to and from cells (36). Apolipoproteins are specific, lipoprotein-associated proteins that control cellular uptake of

lipoproteins by binding to membrane lipoprotein receptors (37). Lipoprotein metabolism includes the exogenous and endogenous pathways as well as the reverse cholesterol transport.

The exogenous lipoprotein metabolism pathway

In the enterocytes absorbed dietary TAGs are incorporated into CMs with apolipoprotein B48 (apoB48) (37), apoAIV and apoAV. They later acquire additionally apoAI, apoAII, and apoC (apoCI, apoCII and apoCIII), and finally, apoE. Functions of apolipoproteins are class- and isoform-specific. ApoB84, apoB100 and apoE function as receptor ligands (36), whereas apolipoprotein AI, the best characterized of its class, is an activator of the enzyme lecithin-cholesterol acyl transferase (LCAT). ApoCII is an essential activator (36) and apoCIII an inhibitor of LPL (37).

CM-TAGs undergo intravascular hydrolysis by the insulin-activated LPL (38), resulting in FFA delivery to cells. Various apolipoproteins are returned to HDL, and, as a result, the size of CMs decrease and they become CM remnants. These are subsequently taken up by the liver after apoE binds to the lipoprotein receptor-related protein (LRP) or the LDL receptor (39).

The endogenous lipoprotein metabolism pathway

In the endogenous pathway, TAGs are secreted from the liver in VLDL particles, which contain apoB100 and subsequently acquire apoA, apoC, and apoE (37) mostly from HDL. In the periphery VLDL-TAG is hydrolysed by LPL transforming the VLDL into remnants (40), which in turn bind the hepatic LDL receptor. The apolipoproteins lost in the process are transferred to HDL. Binding of the VLDL particles is accompanied by further hydrolysis by HL, after which the depleted particle may be removed by receptors or converted into LDL (41), which remains in the circulation. In the blood, both VLDL and LDL lose some of their TAGs to HDL in exchange for cholesteryl esters (42), mediated by cholesteryl ester transfer protein (CETP) (43). HDL is the smallest of the lipoproteins and is responsible for the removal of cholesterol from the peripheral tissues and its transport to the liver for excretion (44, 45), which is sometimes called the reverse cholesterol transport (RCT).

Molecular lipids

In addition to the above-mentioned major lipid species, various smaller groups of lipids, including sphingomyelins, ether-linked lipids, lysophosphatidylcholines, ceramides,

eicosanoids, and lyso-phospholipids, *etc.*, exist in the body and can be identified and quantified by ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-MS) (46). Identification of these lipids is currently a hot topic in lipid and NAFLD research in order to better understand their contribution in health and disease.

2. Non-alcoholic fatty liver disease (NAFLD)

2.1 Definition and disease spectrum

NAFLD covers a spectrum of disorders ranging from simple steatosis (non-alcoholic fatty liver, NAFL) to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (1, 47).

Steatosis is defined as a condition where the percentage of hepatocytes with macroscopic steatosis when examined by histology exceeds 5-10% (48). When measured by proton magnetic resonance spectroscopy (¹H-MRS), the upper limit of normal was 5.56% in the population-based Dallas Heart Study. This limit corresponded to the 95th percentile of liver triglycerides in subjects (n=345) without excess alcohol consumption, elevated S-ALT or abnormal fasting glucose (49). Liver steatosis is defined as non-alcoholic when it is not due to excess alcohol consumption (daily alcohol intake of <20 g for women and <30 g for men, (50)) or other known causes of steatosis such as hepatitis B and C, toxic, autoimmune, or drug-induced causes, Wilson's disease, or hypolipobeta proteinemia (51).

NASH is a pattern of injury in NAFLD and is distinguished from steatosis, or steatosis with mild inflammation by lobular inflammation and hepatocyte ballooning (see Section 4.1.). Fibrosis is not required for the diagnosis of NASH, although it is often present (52, 53).

2.2 Natural course

Simple steatosis has been thought of as more of a benign condition, but this view has been recently challenged, as paired biopsy studies have shown that NAFL can progress to NASH and fibrosis (54).

In a Chinese prospective multicentre study, including 52 biopsied NAFLD patients, after a 3 year follow-up, of the 13 patients with simple steatosis at baseline, 15% had normal liver, 23% continued to have steatosis, 39% developed borderline NASH, and 23% developed NASH (55). Among those with NASH at baseline (n=17), only one patient (6%) regressed to simple steatosis. There was no significant change in anthropometric parameters and lipid

profile in the follow-up (55). In another study with paired biopsies (n=40), after a median 11 year interval, none had developed NASH (56). In 108 biopsied patients referred to a tertiary NAFLD clinic, during a median of 7-year follow-up progression to NASH was seen in 44% of patients with baseline NAFL (54). In this cohort, the BMI had also significantly increased and patients were more likely to have diabetes (54). In 106 NASH patients with paired-biopsies, after a median of 4.3 years one-third showed progression of fibrosis (57).

NAFLD increases the risk of hepatocellular carcinoma (HCC) (48). This occurs especially in patients who have developed liver cirrhosis (58), but cirrhosis is not necessary for HCC to develop. The yearly risk of progression from the final stage of fibrosis (stage 4, cirrhosis) into HCC is 2-3% (59). Worryingly, a study in 49 biopsied HCC patients showed that simple steatosis may also progress to HCC without cirrhosis (60).

2.3. Epidemiology

2.3.1 NAFLD

NAFLD is the leading cause of chronic liver disease in the Western world (61), and it is projected to soon become the leading cause of liver transplantation (62).

Prevalence estimates of NAFLD based on measurement of liver fat content vary depending on the population examined (i.e. ethnicity, sex, comorbidities) and the sensitivity of the diagnostic test applied (63). Using ¹H-MRS in an unselected population of 2,339 U.S. adults in the Dallas Heart Study, one-third (31%) were found to have hepatic steatosis. Ultrasound studies assessing steatosis in Europe have shown the prevalence to range between 20% and 33% (64-66).

Biopsy-based studies in healthy living liver donors showed the prevalence of simple steatosis (NAFL) to be 12-37% in Europe (67, 68) and 26-31% in the U.S. (69, 70). A post-mortem biopsy study in an unselected population of 498 subjects aged 3-94 years in Greece revealed NAFL in 31% (71).

A large meta-analysis based on imaging of steatosis found the prevalence of NAFLD to be 23% in Europe, 24% in North America, 30% in South America, 32% in the Middle East, 27% in Asia, and 13% in Africa (12). In the same meta-analysis, the pooled global prevalence of NAFLD was estimated to be 25% (12).

Prevalence of NAFLD in relation to metabolic risk factors

In patients with NAFLD, the most common cause of death is cardiovascular disease (CVD) (72, 73). The rate of CVD mortality in subjects with NAFLD is approximately 2-fold higher than in those without NAFLD (74). In addition, liver-related mortality is up to six times as common in patients with NASH than in non-NASH subjects (73, 75).

The prevalence of metabolic risk factors is greatly increased in NAFLD. In the DIONYSOS study, NAFLD diagnosed by ultrasound (US) was present in 94% of obese (BMI>30 kg/m²), in 67% of overweight (BMI 25.0-29.9 kg/m²), and in 25% of normal-weight subjects (BMI 20.0-24.9 kg/m²) (76). In severely obese subjects, the prevalence is even higher, 85-98%, in studies based on liver biopsy (77-80). Similarly, patients with type 2 diabetes (DM2) have a much greater overall prevalence of NAFLD (40-70%) than the general population (81-85). A recent study in 100 DM2 patients, in which NAFLD was measured using ¹H-MRI and fibrosis by magnetic resonance elastography (MRE) found the prevalence of NAFLD to be 65% and that of advanced fibrosis 7% (86).

2.3.2 Non-alcoholic steatohepatitis (NASH)

Biopsy is the golden standard in distinguishing between simple steatosis and NASH, and thus the current NASH prevalence estimates rely largely on selected groups of biopsied subjects. In a study with biopsies taken because of elevated transaminases due to unknown causes NASH prevalence was found to be 16% (87). In healthy liver donors, the corresponding proportion was 6-15% (67, 70). In subjects who had fatty liver based on ultrasound, NASH was found in 30% (85, 88). An earlier (1990) autopsy study in Canada found the prevalence of NASH to be 3% (89), while a post-mortem study in Greece found NASH in 40% of subjects (71). In the latter study, nevertheless, the patients had a mean age of 65 years and had metabolic comorbidities (half of them died of coronary artery disease). Alarming, in patients evaluated for liver transplantation, the prevalence of NASH showed an 8-fold increase from 1.2% to 9.7% in just ten years (90, 91). A systematic review from 2011 estimated the prevalence of NASH to be 3-5% (58). There are, however, no population-based estimates of the prevalence of NASH.

Prevalence of NASH in relation to metabolic risk factors

In the aforementioned Canadian autopsy study, NASH prevalence was found to be 18.5% in

markedly obese subjects, and a history of type 2 diabetes (DM2) was associated with a 2.6-fold increase in NASH (89). NASH is estimated to be present in 25-30% of obese or diabetic patients, and in 35% of severely obese patients with DM2 (92-94). In patients biopsied during bariatric surgery, the prevalence estimates of NASH have ranged from 23% to 56% (79, 80, 95-97).

A pooled overall NASH prevalence estimate in a meta-analysis of biopsied NAFLD subjects was 59%. The pooled regional NASH prevalence estimates in NAFLD patients with an indication for biopsy were 63% for Asia, 69% for Europe, and 60% for North America (12).

Fibrosis

NASH affects progression of fibrosis. In a meta-analysis, liver fibrosis progressed by one stage over 14 years in patients with NAFLD and by the same amount in only 7 years in patients with NASH (98). Diabetes increases the risk of fibrosis development (54), as does older age and metabolic abnormalities (56). A recent study examining the changes in prevalence of hepatitis C (HCV), NASH, and alcoholic liver disease (ALD) among patients with cirrhosis or liver failure from three different databases (NHANES, HealthCare Integrated Research database, UNOS, between the years 2003 and 2015) found decreases in percentages of patients with compensated cirrhosis from HCV or ALD, but an increase in percentages of patients with cirrhosis from NASH (99). A similar trend was seen in chronic liver failure (CLF), with an almost 3-fold increase in proportion of patients with CLF from NASH (99). This increase indeed parallels the increase in prevalence of obesity.

HCC

NAFLD has been suggested to have become the most common liver disease associated with HCC. In 2010, NAFLD was the most common (35%) cause of referral to the hepatology clinic amongst patients with HCC (100). Obesity (101) and diabetes (102) are also known risk factors for HCC.

There is a worrying trend in the incidence of HCC. In the U.S., the number of cases with HCC increased by 115% in just 12 years (2000-2012) and the age-adjusted incidence increased 1.5-fold from 4.4/100 000 in 2000 to 6.7/100 000 in 2012 (103). In the past 50 years, the age-adjusted incidence of HCC in Finland has increased 5-fold in men and 2-fold in women (104).

2.4 Risk factors

2.4.1 Age, gender, and ethnicity

NAFLD increases with age (105, 106). Age together with metabolic risk factors identifies patients with NASH who are at a high risk of advanced liver fibrosis (107, 108). Nevertheless, the higher prevalence and disease severity could be a result of a cumulative effect of longer duration of NAFL/NASH and an increase in the prevalence of components of metabolic syndrome.

Gender. Available data on the role of gender in predisposition for NAFLD is conflicting. Although earlier reports suggested NAFLD to be more prevalent in women than in men (95, 109, 110), several more recent studies have found the opposite (65, 85, 106). Consistent with these studies, men have more intra-abdominal and liver fat than women with the same BMI (2, 111).

Ethnicity. The role of ethnicity in NAFLD is well established. In the U.S., NAFLD was found to be most common in Hispanics (45%), followed by European Americans (33%) and lastly African Americans (24%) (112). This finding has been supported by a recent large study (N = 9675) on the racial differences in NAFLD (113), as well as a large meta-analysis (12). Hispanics also have a higher prevalence of NASH and cirrhosis than other ethnic groups, whereas African Americans are less likely to develop liver failure (114).

As the racial variations can only partly be explained by the differences in ethnic lifestyle the role of genetic predisposition is suggested. This is supported by a study in which the *PNPLA3-I148M* allele was predominant in Hispanics, the group most prone to developing NAFLD (6).

2.4.2 Obesity, metabolic syndrome and type 2 diabetes

Obesity. Increasing obesity parallels the incremental burden of NAFLD, especially in the Western countries, and it is the most important risk factor for NAFLD (77, 115). In the U.S., more than one-third of the population is obese (116) and roughly the same proportion has NAFLD (112). BMI and waist circumference both positively correlate with the presence of NAFLD (117). Nevertheless, NAFLD can be present in individuals with normal BMI and without metabolic risk factors (85, 118).

Metabolic syndrome. The liver, once fatty and insulin-resistant, overproduces components of metabolic syndrome (112, 119, 120), which is why the conditions are tightly interrelated and NAFLD is sometimes referred to as the hepatic manifestation of metabolic syndrome (121). Like metabolic syndrome, NAFLD also increases the risk of both type 2 diabetes and cardiovascular disease (122).

Type 2 diabetes. Several longitudinal studies have shown NAFLD to predict type 2 diabetes (74), and patients with type 2 diabetes are strongly predisposed to NAFLD (82, 84). This association is observed even in the absence of obesity (119). Type 2 diabetes has been suggested to be a key factor in the progression of NAFLD to NASH, fibrosis and HCC (102, 123, 124).

2.4.3. Dietary factors and exercise

Diet. There is a lack of long-term intervention studies comparing effects of diet on NAFLD, but based on the available knowledge some conclusions are justified.

Hypocaloric diets decrease and caloric excess increase liver fat content (125). Low-fat and high-carbohydrate diets seem to decrease liver fat relative to high-fat and low-carbohydrate diets of similar caloric intake (126-128). The negative effect of high-fat diets appears to be due to SAFA (129). On the contrary, PUFA or MUFA diets may be beneficial (130, 131). A recent meta-analysis on the effect of different amounts of dietary fructose on the prevalence of NAFLD concluded that the harmful effect of fructose might be attributed to high calorie intake (132).

Exercise. The benefit of exercise is unquestionable in NAFLD patients. Both aerobic and resistance exercise can improve NAFLD (133-136), even in the absence of significant weight loss (137). NAFLD is also associated with a low level of physical activity (138). A very recent systematic review comparing the benefits of resistance training with aerobic training in NAFLD patients found both to be effective in improving hepatic steatosis (139). The writers summarized the median effective exercise protocols to be moderate (4.8 METs) aerobic training sessions of 40 min/session 3 times a week, and for resistance training 3.5 METs for 45 min/session 3 times a week (139). The latter is in concordance with the EASL-EASD-EASO clinical practice guidelines (47).

2.4.4 Genetics

There is a substantial heritable component to NAFLD across all ancestries (140). In individuals with Hispanic ancestry, the heritability of population-based NAFLD was found to be between 31% and 38% (141, 142). In three European ancestry family-based studies (Family Heart Study, The Old Order Amish, and the Framingham Heart Study), heritability was calculated to range from 26% to 27%. A population-based twin study in Finland suggests approximately 60% of the variation in ALT is genetically determined (143). In a U.S. cohort of 60 pairs of twins, the heritability of hepatic steatosis was 52%, and that of fibrosis 50% (144).

Also, the difference in NAFLD prevalence in different regions can only partially be explained by differences in *e.g.* obesity, suggesting genetic differences in the predisposition to develop fatty liver (6, 145). As discussed above, NAFLD correlates with metabolic abnormalities, but imperfectly so (146).

2.4.4.1 GWAS

To identify specific genetic factors that are associated with NAFLD, several genome-wide association studies (GWAS) have been carried out. The first exome analysis of hepatic steatosis measured by ¹H-MRS was performed in 2008 by Romeo *et al.* (6) who found that a variant in the *PNPLA3* gene, the G-allele at rs738409, encoding an I148M missense mutation, markedly and highly significantly contributed to increased liver fat content across several ethnic groups in the U.S. The same study, together with a more recent one (145), found that the difference in frequency of the *PNPLA3* effect allele across ancestries accounted for more than 70% of the global variation in the prevalence of NAFLD (6, 145). Subsequent studies have replicated the association of the same variant not only with hepatic steatosis, but also with increased ALT activities (8, 147-149), steatosis diagnosed by imaging techniques (150-152), and abnormal liver histology, including steatosis, NASH, and fibrosis/cirrhosis (4, 7, 9, 151, 153, 154). A recent meta-analysis of almost 3000 histologically determined NAFLD subjects showed that individuals homozygous for the *PNPLA3* gene variant had 73% higher liver fat content, 3.24-fold greater risk of necroinflammatory scores, 3.2-fold greater risk of developing fibrosis, and 3.44 higher odds of developing NASH than those lacking the gene variant (155).

The search for genetic factors underlying NAFLD is ongoing and various candidate genes in

addition to *PNPLA3* have been described. One of the largest GWAS studies using computer tomography (CT) to diagnose NAFLD (n=7176) and ~2.4 million SNPs suggested that additional variants in or near *PNPLA3*, *NCAN/TM6SF2*, *LYPLAL1*, *GCKR* and *PPP1R3B* genes influence liver fat content (151). These top variants associated with steatosis were assessed for their effects on 592 cases of biopsy proven NASH/fibrosis, and all of the variants except *PPP1R3B* were also found to be associated with progression of the disease (151). The association of the variants *TM6SF2* and *GCKR* with NAFLD has been confirmed by subsequent independent studies (156-158).

One of the most recent genes to be discovered in 2015 was a variant in membrane-bound O-acyltransferase domain containing 7 (*MBOAT7*) at rs641738 (159). It was shown to increase the risk of alcohol-related cirrhosis (159) and has since been additionally shown to increase the risk of steatosis and histologic liver damage in NAFLD, independent of obesity (160).

2.4.4.2 *PNPLA3*

The *PNPLA3* gene resides on chromosome 22 and stands for patatin-like phospholipase domain-containing protein 3, also called adiponutrin. In 2008, a single-nucleotide polymorphism (SNP) at rs738409 was found to cause a substitution from cytosine (C) to guanine (G), resulting in a switch from isoleucine to methionine at position 148 (I148M) (6). This variant has since been associated with liver fat and progressive liver disease (see below).

PNPLA3 gene encodes for a protein that relates structurally to the principal TAG hydrolase in adipose tissue, adipose tissue triglyceride lipase (ATGL/PNPLA2) (161, 162). In humans, it is predominantly expressed in the liver and distributed between membranes and lipid droplets (163). In vitro studies have demonstrated the wild-type enzyme to have hydrolytic (lipolytic) activity against three major glycerolipids (tri-, di-, and monoacylglycerides), with a strong preference for those with oleic acid (C18:1) as their acyl moiety (164, 165). The I148M amino acid substitution affects the lipophilic substrate-binding site of the hydrolase, and thus, may prevent the binding of substrates to the catalytic site (165). Tracer studies and in vitro models have demonstrated reduced VLDL-TAG secretion in the I148M carriers, which could suggest failure to mobilize TAGs from intracellular droplets due to the diminished hydrolase action *i.e.* loss-of-function (166). This model has nevertheless been challenged by, for example, the finding that deletion of *pnpla3* in mice did not initiate hepatic steatosis (167).

In line with the latter, in addition to lipase activity, *PNPLA3* has been demonstrated to exhibit lysophosphatidic acid acyltransferase (LPAAT) activity, which promotes intracellular lipid synthesis by converting LPA to phosphatidic acid (168). In accordance with this, the human I148M rs738409 variant had increased LPAAT activity (168). This gain-of-function mutation causing increased intracellular lipid synthesis is an additional suggested mechanism for hepatic lipid accumulation in the variant carriers (168). Further studies have confirmed that overexpression of the *PNPLA3* I148M exclusively in the liver leads to hepatic steatosis, with three notable metabolic effects: increased synthesis of FAs and TAGs, reduced TAG hydrolysis, and relative depletion of TAG long-chain PUFAs (169).

Mice studies have shown that the metabolic milieu may affect *PNPLA3* expression, which is reduced by fasting and increased after feeding (170, 171). Postprandial *PNPLA3* expression is controlled by insulin via liver X-receptor-retinoid X-receptor (LXR-RXR) and sterol regulatory element-binding protein 1 (SREBP-1), and additional posttranslational control is provided by specific FAs (such as palmitate C16:0, oleic acid C18:1, linoleic acid C18:2) by increasing *PNPLA3* expression (163).

PNPLA3 and the spectrum of NAFLD

Due to the implication that *PNPLA3* mutation is a possible modifier of NAFLD pathogenesis, the polymorphism has been extensively studied and its association with the accumulation of liver fat independently confirmed in numerous candidate gene studies (5, 7-9, 154). Also, an association with elevated AST and ALT levels has been established (152), and there is increasing evidence that the rs738409 (I148M) variant is associated with more severe NASH and greater fibrosis (7, 9, 154), as well as an increased risk of HCC (172, 173). Curiously, NAFLD patients with the *PNPLA3* mutation (“*PNPLA3* NAFLD”) present with less metabolic abnormalities, such as hyperglycemia, hypertriglyceridemia, and low HDL cholesterol concentration, than NAFLD patients with the wild-type *PNPLA3* (“obese/metabolic NAFLD”) (3). Additionally, ‘*PNPLA3* NAFLD’ dissociates from insulin resistance (8) and type 2 diabetes (6), and seems to be more susceptible to a weight loss-induced decrease of hepatic fat (174-176). These latter findings support NAFLD aetiology as heterogeneous and the existence of NAFLD subtypes.

Prevalence of PNPLA3 variant

The prevalence of *PNPLA3* rs738409 is dependent on the population studied. In Western

populations, the prevalence of the heterozygous (CG) gene variant is 35-42% and the homozygous (GG) gene variant about 5% (177). In Finland, the prevalence of the CG variant is around 36% and that of the GG around 6% (5).

2.4.4.3 TM6SF2

After the current studies, an exome-wide association study in over 2700 subjects identified a nonsynonymous SNP (rs58542926) in the *TM6SF2* gene to be associated with fatty liver disease (156). Subsequently, the E167K variant has been robustly associated with steatosis, NASH, and advanced fibrosis/cirrhosis (151, 156, 178-182).

Transmembrane 6 superfamily member 2 (*TM6SF2*) is a gene encoding a 351 amino acid protein with hitherto unknown function (182). It is expressed predominantly in the liver and intestine (182). The *TM6SF2* rs58542926 variant is characterized by a C-to-T substitution in nucleotide 499, encoding a glutamate to lysine substitution at codon 167 (E167K). In vitro (156, 180, 182) and in vivo (179, 180) studies suggest that the *TM6SF2* gene mutation or deletion results in reduction of lipoprotein secretion (VLDL, TAG, and apoB) with a corresponding increase in hepatocellular lipid droplet size and TAG content.

The *TM6SF2* rs58542926 variant provides new insight into the pathogenesis of NAFLD and into the association between NAFLD and cardiovascular disease. The metabolic effect of the mutation suggests that the *TM6SF2* could act as a 'switch': the rs58542926 T-allele causes hepatic retention of TAG and cholesterol, predisposing its carriers to NAFLD fibrosis, whereas the C-allele (wild-type) is liver protective by promoting VLDL excretion, but at the cost of increased risk of cardiovascular disease (140).

The prevalence estimate of the minor allele is 7% in Europeans, 5% in Hispanic Americans, and 3% in Africans (156).

3. Pathogenesis of NAFLD

Circulating lipids. The circulatory FFA pool represents a major source of hepatic fat accumulation in NAFLD patients. Due to the inhibitory action of insulin on lipolysis, FFA concentrations are lower after eating and high during fasting. When adipose tissue becomes

insulin-resistant (IR), it also becomes resistant to the anti-lipolytic effect of insulin, resulting in high FFA concentrations despite high insulin concentration (183, 184). The circulating FFAs are reabsorbed by various organs (adipose tissue, liver, muscle) and some will be oxidized, whereas the remaining FFAs will accumulate as intracellular TAG droplets and lipid intermediates (such as DAGs and ceramides). The efflux of lipids from the liver happens via TAG-VLDL, which results in lowering of HDL cholesterol and an increased risk of atherosclerosis (185).

Pathways of intrahepatocellular triglyceride synthesis. Studies using stable isotopes have shown the majority of liver fat to be derived from the circulating FFA pool (59%), and the rest to originate from increased DNL (26% in fatty liver vs. ~5% in normal-weight subjects), as well as dietary FAs (15%) (24). The development of peripheral insulin resistance results in failure of insulin to inhibit release of FFA from adipose tissue, leading to increased FFA delivery to the liver (186, 187). Hyperinsulinemia also stimulates DNL via stimulation of regulatory transcription factors (SREBP-1c and ChREBP) (188). Lack of insulin inhibition of gluconeogenesis is also responsible for increased DNL, as more metabolites are directed to DNL instead of glucose production, at least in mice (189).

Mechanisms of lipid-induced insulin resistance. FFAs promote insulin resistance via substrate competition (190) and by inhibiting insulin signalling pathways. The latter occurs via bioactive lipids such as ceramides (191) and diacylglycerols. Ceramides are formed from saturated fatty acids and inhibit insulin-induced Akt/PKB phosphorylation (192). They also induce ER and oxidative stress (192) and are proinflammatory via activation of the c-Jun N-terminal kinase (JNK) pathway, which induces hepatic fat accumulation and insulin resistance in mice (192). We recently showed increased hepatic ceramides, saturated TAGs and FFA characterize the liver lipidome in obese NAFLD (11). They also activate pro-inflammatory M1-macrophages (193). DAGs have been suggested to cause lipid-induced insulin resistance via PKC- ϵ -mediated insulin receptor Thr160 phosphorylation (194).

3.1 NAFLD

3.1.1 ‘Obese/metabolic NAFLD’

Obesity is common in metabolic syndrome and NAFLD, even though both can exist in non-obese individuals. Metabolic syndrome (MetS) develops especially in those obese individuals

with accumulation of fat in the liver ('obese NAFLD') (Fig. 1). Insulin resistance is the hallmark of this type of NAFLD, leading to hyperglycemia, hyperinsulinemia, increased production of VLDL and serum TAGs, which lowers HDL cholesterol (3). DNL is similarly increased in hyperinsulinemic subjects, as the stimulatory effect of insulin on DNL remains insulin-sensitive (195). Caloric excess due to overeating increases hepatic FA influx via peripheral lipolysis and hepatic lipogenesis, resulting in a fatty liver.

Adipose tissue. Adipose tissue of 'obese NAFLD' individuals show several changes, including hypoxia (196), increased infiltration of macrophages surrounding dead adipocytes, and expression of chemokines and proinflammatory cytokines (197). Inflamed adipose tissue is insulin-resistant, leading to increased lipolysis and FFA flux to the liver (198). There is also a deficiency of the insulin-sensitizing and anti-inflammatory molecule adiponectin, which could contribute to fat accumulation in the liver and induce hepatic inflammation and insulin resistance (199). Adiponectin deficiency also contributes to increased ceramide concentrations by impairing their degradation (192).

Gut-liver axis. A relatively new area of research is investigation of the role of gut microbiota and its changes in both obesity and NAFLD (200). Altered permeability of the intestinal mucosa to inflammatory mediators, such as endotoxin, is hypothesized to have a role in NAFLD pathogenesis.

3.1.2 'PNPLA3 NAFLD'

In contrast to 'obese NAFLD', metabolic abnormalities do not on average characterize NAFLD associated with *PNPLA3* rs738409 ('PNPLA3 NAFLD') (Fig. 1). Thus, as discussed earlier (Review of the Literature, Section 2.4.4.2 *PNPLA3*), 'PNPLA3 NAFLD' is not accompanied by insulin resistance, hyperglycemia, hypertriglyceridemia and low HDL cholesterol (5, 6, 151, 201-204), or inflammation in adipose tissue (205). Unlike previous studies that have shown *PNPLA3* mutation to increase liver fat, whilst having no effect on body weight, a recent study by Park *et al.* (206) showed liver fat to differ between individuals with different *PNPLA3* genotypes (wild-type vs. mutation) but similar BMI. In fact, when adjusting BMI with liver fat in the groups with different genotypes, carriers of the gene variant ('PNPLA3 NAFLD') were leaner than those in which NAFLD was due to obesity ('obese NAFLD') (207).

The mechanism behind the *PNPLA3* I148M variant induced liver fat accumulation has been

discussed in more detail in Section 2.4.4.2 *PNPLA3*. Studies in mice have shown overexpression of the human *PNPLA3* I148M variant exclusively in the liver to cause hepatic TAG accumulation (169). Overexpression of the same variant in adipose tissue had no effect on liver fat content, body weight, fat distribution, or adipose tissue TAG content, morphology or gene expression (169). Individuals homozygous (GG) to I148M variant allele show a lower rate of DNL (which produces IR-inducing SAFAs) than those without the gene variant (208). In contrast to ‘obese NAFLD’, where there is an increase in hepatic ceramides, saturated TAGs, and FFA, the ‘*PNPLA3* NAFLD’ predominantly contains an excess of polyunsaturated TAGs, with no changes in FFA (11). In vitro studies have found the I148M gene variant to decrease the rate of lipolysis of intrahepatocellular TAG (169), whereas subsequent studies found the mutation to increase TAG synthesis (168). In line with the gain-of-function theory, a recent study by Luukkonen *et al.* (11) found an increase in hepatic polyunsaturated TAGs and DAGs in the I148M variant allele carriers. Nevertheless, our current understanding of whether the I148M *PNPLA3* variant inhibits TAG lipolysis or stimulates TAG synthesis or both is still limited and conclusions require further research.

Figure 1.

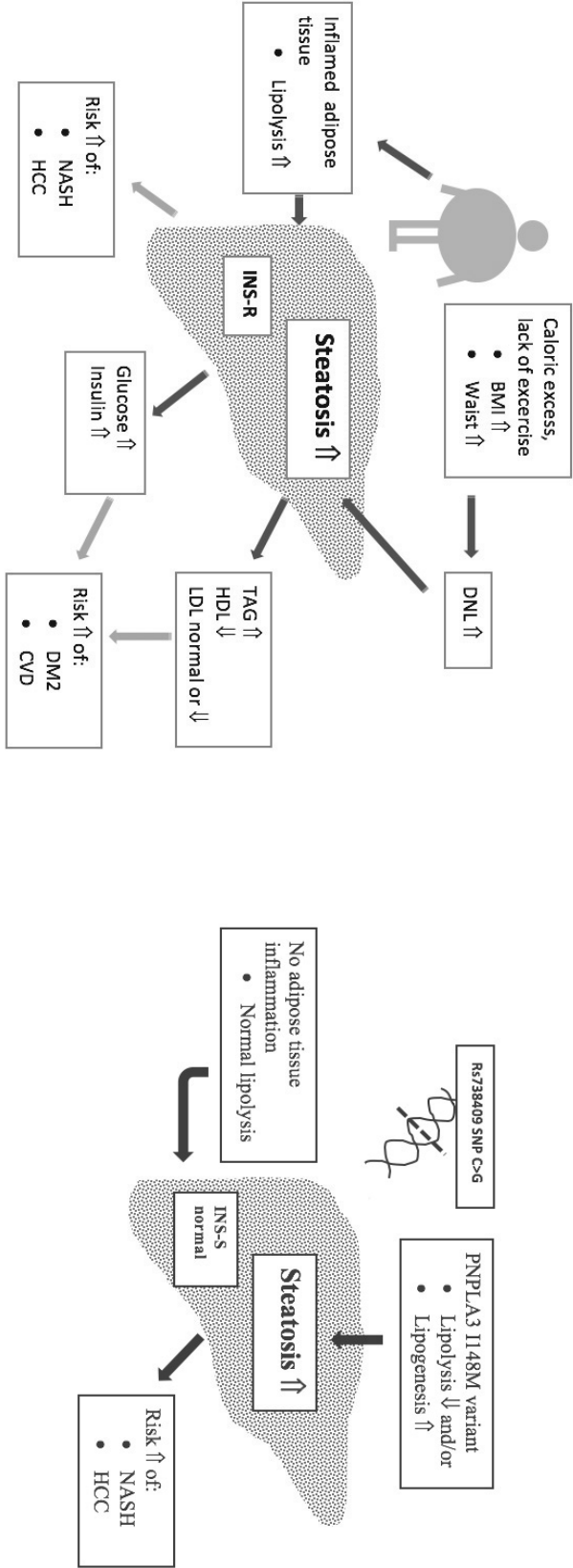


Figure 1. Obese/ metabolic NAFLD (left) vs. PNPLA3 NAFLD (right). Adapted from Yki-Järvinen and Luukkonen (209)

In 'obese NAFLD', caloric excess from overeating increases hepatic FA influx via peripheral lipolysis and hepatic lipogenesis, leading to fat accumulation in the liver. The liver in this type of NAFLD is insulin-resistant, causing metabolic abnormalities and increasing the risk of cardiovascular disease and type 2 diabetes. On the other hand, in 'PNPLA3 NAFLD' the I148M variant impairs hepatic lipolysis and/or increases lipogenesis, leading to hepatic steatosis without causing insulin resistance or increasing the risk of cardiovascular disease or type 2 diabetes.

3.2. NASH

In NAFLD, accumulation of TAGs in hepatocytes could be viewed as an early adaptive response to convert potentially lipotoxic FFAs into metabolically inert TAGs (210). Increased DNL promotes accumulation of lipotoxic intermediates, such as DAGs and ceramides, which cause IR (192, 211). Increased FFA influx, together with reduced TAG efflux from the liver is believed to overpower the adaptive mechanisms promoting hepatocyte lipotoxicity, generation of reactive oxygen species (ROS), and endoplasmic reticulum ER stress (74, 212). These disturbances combine with the harmful effect of cytokine release by Kupffer cells and immune-mediated cellular injury (213-215). Kupffer cells are resident macrophages that release cytokines, such as IL-1 β and TNF- α , in response to messages from injured cells and TLR4, leading to inflammasome activation (216-219). Hepatocellular damage and stimulation of cell death pathways mark the transition to steatohepatitis (NASH).

It remains unclear why some individuals but not others with simple steatosis develop NASH. Progression of NAFLD/NASH is likely to reflect the combined effect of several molecular and immunological processes and is a “multiple hits” rather than a “first hit – second hit” phenomenon.

Lipotoxicity. Hepatic SAFAs are mainly derived from adipose tissue, the diet and *de novo* lipogenesis. Normally, SAFAs are transported to mitochondria for β -oxidation or esterified for either excretion in VLDL or storage as TAG. When the liver is overwhelmed by SAFA, multiple mechanisms could cause liver injury in hepatocytes (220). These lipids stimulate a variety of intracellular responses, especially formation of ceramides (11, 192), resulting in lipotoxic stress in the ER and mitochondria (221). Consequently, apoptosis occurs – a key pathogenic feature of NASH.

Oxidative stress and mitochondrial dysfunction. FA oxidation in liver is the main source of ROS. Oxidative stress arises from an imbalance between production of ROS and that of antioxidants, and represents a common final pathway in FFA lipotoxicity (222). Normally, short-, medium-, and long-chain FAs undergo β -oxidation primarily in the mitochondria of the liver via the tricarboxylic acid (TCA) cycle (223). But when there is an excess of FFAs, minor pathways, such as β -oxidation in the peroxisome, and cytochrome-mediated ω -oxidation in the ER, become relevant, leading to an increased production of hepatic ROS (223). In line with this, both peroxisomal β -oxidation and ω -oxidation of long-chain fatty

acids are increased in NASH (224). Oxidative stress results in nuclear and mitochondrial DNA damage, phospholipid membrane disruption by lipid peroxidation, and the release of proinflammatory cytokines (223).

Switching from mitochondrial to peroxisomal/ER oxidation could be a result of mitochondrial dysfunction (225) or abnormal morphology (222). A recent study discovered mitochondrial function to be increased in obese individuals with or without fatty liver, relative to lean individuals, despite similar hepatic mitochondrial mass (226). On the other hand, individuals with NASH had higher mitochondrial mass, but up to 40% lower maximal respiratory function, than obese individuals with or without fatty liver (226). This indicates ability of mitochondria to adapt in the early stages of obesity and simple steatosis, which is subsequently lost in NASH.

Endoplasmic reticulum stress. Lipid accumulation and impaired lipid oxidation can lead to dysfunction of the ER. The ER is a membranous intracellular network responsible for folding of majority of secreted and membrane proteins, synthesis of PLs, and functioning of enzymes, including cytochrome P450 (227, 228). Not only can obesity (229) and hepatic steatosis (230) induce ER stress, but experimental models have shown ER stress *per se* to be able to induce hepatic steatosis (231), as well as to inhibit VLDL secretion (232). Together these changes form a vicious circle of fat accumulation in the liver.

ER stress may also lead to misfolding or unfolding of proteins. These proteins form aggregates triggering the unfolded protein response (UPR) pathway, which aims to re-establish normal homeostasis by decreasing protein synthesis and ER-associated protein degradation (233). If this response is inadequate, stress sensor proteins, such as transcription factor 6 (TF-6) and inositol-requiring enzyme-1 (IRE-1) can trigger apoptosis (234, 235). Variable degrees of UPR activation are seen in both NAFLD and NASH (234).

Other factors. In addition to the above-mentioned mechanisms, many additional “hits” have been identified to contribute to NASH pathogenesis. To name a few; innate immunity and gut-derived signals, with endotoxin (LPS) and TLR4 as important components, may promote NASH (223). Chemokines and their receptors, such as C-C chemokine receptor 2 and its ligand CCL2, act as major regulators of macrophages in liver during inflammation (236). Nuclear receptors, including liver X receptor (LXR), responsible for regulating cholesterol homeostasis (237), as well as bile acid metabolism-mediating farnesoid X receptor (FXR)

have both been both associated with NASH pathogenesis (238). Autophagy, in which cell constituents are targeted to lysosomes for degradation, is linked to IR, hepatic injury, and fibrogenesis (207) and is suggested to be increased in NASH. Other areas of interest include the role of microRNAs, metabolic hormone pathways, and intestinal microbiota in the progression of NAFLD/NASH.

Hepatocyte injury and death. Ultimately, the “multiple hits” combine to cause cellular damage and induce cell death pathways. Breakdown of cellular components can occur either in a programmed manner with intact plasma membrane (apoptosis) or accidentally in a lytic and inflammatory process (necrosis). Apoptosis is highly regulated and is a prominent feature in NASH (239). Indeed, cleaved cytokeratin 18 fragments (CK18), markers of apoptotic activity, have been correlated with NAFLD disease severity (240). Additionally, death receptors responsible for initiating apoptosis, such as Fas and TNF-receptor1, are increased in livers of NASH patients (239, 241). These cell death pathways remain an intense area of research and could potentially be used as markers of disease progression in NAFLD.

PNPLA3 and NASH. Previous studies have shown the I148M *PNPLA3* variant to predispose to NASH independent of features of IR (155). In a recent study (11), subjects with ‘Metabolic NAFLD’ and ‘PNPLA3 NAFLD’ had an equal frequency of NASH despite different hepatic TAG and bioactive lipid compositions. Fat accumulation in the liver without hepatocellular injury predicts NASH (54), and thus, the *PNPLA3* variant could simply increase the risk of NAFLD by increasing steatosis.

On the other hand, *PNPLA3* is highly expressed in human hepatic stellate cells (HSCs), and its expression seems to be regulated by retinol availability and insulin. Increased *PNPLA3* expression resulted in reduced lipid droplet content, whereas mutation in the *PNPLA3* was accompanied by lower levels of retinol binding protein 4 (RBP4), indicating an intracellular retinol retention due to a loss-of-function in this *PNPLA3* variant (242). Thus, possible links between *PNPLA3*, retinol metabolism and progressive NAFLD have also been suggested.

4. Diagnosis of NAFLD

4.1 Biopsy

NAFL *i.e.* simple steatosis. Hepatic steatosis is defined as a condition where at least >5% of hepatocytes are lipid-filled (48) (Table 1). Steatosis in NAFLD is typically macrovesicular, in which a single or several well defined cytoplasmic lipid droplet(s) displace the nucleus to the cell periphery (243). Microvesicular steatosis, in which lipid droplets in the cytoplasm are minute and localized around a centrally placed nucleus, may also occur. A mixture of the two types, micro- and macrovesicular steatosis is often observed (243). Additionally, in simple steatosis foci of lobular inflammation, mild portal inflammation and lipogranulomas but not features of hepatocellular injury and fibrosis can also be seen (243). Fat accumulation tends to start in the acinar zone 3 *i.e.* in the perivenular area of the hepatocyte. Thus, the extent of steatosis is mainly evaluated from this area and graded as mild (0-33%), moderate (33-66%), or severe (>66 %). In severe steatosis, the whole acinus can be occupied (244).

NASH. The minimal criteria to diagnose histological NASH include steatosis and hepatocyte injury, which usually presents as ballooning and lobular inflammation, typically also in acinar zone 3. Fibrosis is not necessary for diagnosis of NASH (53, 245) (Table 1).

Ballooning degeneration *i.e.* presence of enlarged apoptotic hepatocytes, is the key feature for differentiating NAFL from NASH (246). Ballooning is graded as minimal (0), present (1), or marked (2).

Lobular inflammation is usually mild, consisting of inflammatory cells, including lymphocytes, some eosinophils, and sometimes a few neutrophils. Diffuse lobular aggregates of Kupffer cells *i.e.* microgranulomas and lipogranulomas, are common (247). Portal mononuclear cell infiltration is common and usually mild, and an increased portal inflammation is a marker of severe disease (248).

Fibrosis is not required for the diagnosis of NASH, but its presence is not uncommon (Table 1). Deposition of collagen and other extracellular matrix (ECM) fibres along the sinusoids of especially zone 3 and around the hepatocytes has a characteristic “chicken wire” pattern. In addition, portal fibrosis may occur, and in advanced disease, progression into bridging

fibrosis and cirrhosis develops (244). Sinusoidal collagen formation in NASH is likely to be the result of hepatic stellate cell (HSC) activation (249, 250). Cirrhosis in NASH is usually macronodular or mixed, but not micronodular (243).

Other histological lesions seen in NASH include Mallory-Denk bodies (MDBs) (251), which are associated with the severity of NASH (244) and strengthen the diagnosis, but are not specific to NASH (243). MDBs contain various misfolded proteins, including CK-18 (252). Other findings include megamitochondria, which could be a result of injury from lipid peroxidation or an adaptive change (253). Glycogenated, *i.e.* vacuolated nuclei support non-alcoholic aetiology of steatohepatitis, as they are very rarely seen in alcoholic steatohepatitis (243).

Table 1. Characterization of NAFLD spectrum of diseases. Adapted from Kleiner *et al.* (254).

Histological diagnosis	Definition
Normal liver	Less than 5% of hepatocytes steatotic and no other pathological changes (ballooning, fibrosis) that indicate NASH
Simple steatosis (NAFL)	Steatosis without ballooning or fibrosis
<ul style="list-style-type: none"> • + inflammation • + nonspecific fibrosis 	Possible, non-uniform lobular inflammation and/or mild fibrosis of unknown importance
NASH	Steatosis with zone 3 centred insult pattern including inflammation and ballooning necrosis (often with MDBs), with/without fibrosis.
<ul style="list-style-type: none"> • Borderline NASH of zone 3 	Borderline NASH has some, but not all of the characteristics required for diagnosis of NASH.
Cryptogenic fibrosis/ cirrhosis	Usually advanced fibrosis or cirrhosis, with/ without evidence of steatosis, and no evidence of borderline/definite NASH.

The NAFLD activity score (NAS) was developed to diagnose NASH, originally in intervention studies, by combining histological evaluation of steatosis, ballooning and lobular inflammation (255). $NAS \geq 5$ is suggestive of NASH and < 3 the absence of NASH. Recent

studies have, however, questioned the benefit and prognostic value of the score (256-258). The latest studies support the grading of NAFLD by the SAF -score, in which S represents degree of steatosis (S0: <5%; S1: 5%-33%, S2: 34%-66%, S3: >67%), A the grade of activity (hepatocyte ballooning and activity of inflammatory cells; A0-A4), and F the grade of fibrosis (F0: none, F1: perisinusoidal, F2 perisinusoidal and periportal, F3: bridging fibrosis, F4: cirrhosis) (259). The inclusion of fibrosis is especially important, as it predicts both progression of NAFLD and mortality (72, 260).

A liver biopsy is the only way to diagnose NASH but it cannot be used as a screening tool for population studies due to its high cost, and although a relatively safe procedure, it can have fatal complications, with a death rate of 0.01% (261). Limitations of the biopsy also include sampling error, as only 1/50 000-1/65 000 of the whole liver size is included, and thus, even a good-quality sample of approximately 10 portal tracts represents only a local process in the liver (244). As inflammatory lesions could be unevenly distributed in the liver, a biopsy may wrongly exclude NASH in up to one-third of the cases (262, 263). Additionally, inter- and intra-observer variability represents a serious problem (264), emphasizing the need for analysis by an experienced pathologist.

4.2 Imaging tools

Ultrasound (US) is a readily available tool for identification of steatosis and is the preferred method as a first-line diagnostic test, especially in tertiary settings. It has a sensitivity of 60-94% and a specificity of 66-97% (265-267), but does not reliably detect steatosis when liver fat content is below 20% (268, 269) or in individuals with high body mass index (BMI >40 kg/m²) (70). Despite observer dependency, US robustly diagnoses moderate and severe steatosis and additionally provides important information on focal lesions and the hepatobiliary tract.

Computer tomography (CT) detects fatty liver with a sensitivity of 93% (270) and like MRI, detects focal steatosis better than the US (271). Otherwise the US is more sensitive in diagnosing fatty liver disease (270, 271). However, due to the high radiation dose, CT cannot be used as a screening method (272).

Magnetic resonance imaging and -spectroscopy (¹H-MRI and ¹H-MRS) are both non-invasive, MR-based techniques that are used for determining liver fat content. Both

techniques use the difference in resonance frequencies between water and fat proton signals to quantify the proton density fat fraction (PDFF) (273). PDFF reflects the fraction of mobile protons in liver due to fat. When measured correctly, PDFF is not affected by field strength, manufacturer, patient factors (age, sex, BMI, liver fat aetiology), or concomitant liver abnormalities, such as iron overload or necroinflammation (273). Currently, PDFF is the leading imaging-based biomarker of liver steatosis (273-276).

¹H-MRI can accurately detect liver steatosis of 3% (273) and is a good standard method for diagnosis of fatty liver. However, advances in the ¹H-MRS technique have led to it becoming the golden standard (49); it may be even more reliable than biopsy, as it determines the whole liver volume (277). ¹H-MRS measures a 3D metric of hepatic fat content, *i.e.* the TAG concentration in liver tissue (273), in contrast to histology, which evaluates the number of steatotic hepatocytes (277). Steatosis assessed by the ¹H-MRS strongly correlates with liver biopsy based steatosis grading (278). Also, the sensitivity of ¹H-MRS is excellent and it can detect even small amounts of TAGs, which might be missed on histological analysis (272). Nonetheless the use of ¹H-MRS is still rather limited due to being expensive, not widely available on routine scanners, and requiring special coils and expertise to be used. Thus, the MRI-PDFF modality is currently better for assessment of liver steatosis.

4.3 Circulating markers

AST, ALT. NAFLD is the most common cause for elevated AST and/or ALT in population-based studies in the U.S. (279) and Finland (280), although these enzymes are within the normal range in half of patients with NAFLD diagnosed by ¹H-MRS (2). It is also not uncommon to find normal ALT and AST concentrations in patients with NASH or advanced cirrhosis (281). ALT is more liver-specific than AST, although the AST/ALT –ratio increases once NAFLD progresses. In a study with 238 biopsy-proven NAFLD cases, the area under the ROC-curve (AUROC) for ALT levels was 0.62 for diagnosing NASH (281). Higher cut-off values (53-70 IU/L), as opposed to lower ones (<35 IU/L were more specific (51-61% vs. 29%) but less sensitive (50-72% vs. 89%)) (281). Thus, abnormal liver function tests are neither sensitive nor specific markers of NAFLD. The AST to ALT ratio has also been used to differentiate between alcoholic and non-alcoholic steatohepatitis (282). Nonetheless, a population-based study in 2766 subjects showed no differences in ALT, AST concentrations, or AST/ALT ratio between NAFLD and AFLD (280). Alcohol consumption increases the

gamma-GT concentration markedly and this enzyme reflects AFLD more than NAFLD (280).

Markers of apoptosis. Cytokeratin 18 (CK18) is a major component of intermediate filaments of simple epithelial cells in the liver (283). Liver cell apoptosis is associated with collapse of the keratin cytoskeleton due to activation of intracellular proteases such as caspase-3 and caspase-7 (284), which cleave the intact CK-18 (M65 antigen) into three fragments, one of which is the M30 antigen (285). Both CK-18 and the M30 have been suggested to help in prediction of NASH in a meta-analysis (286), which reported a sensitivity of 83% and specificity of 71% for CK-18 M30 for diagnosis of NASH. The total CK-18 concentration had a sensitivity and specificity of 77% and 71%, respectively (286). Nevertheless, the sensitivity of CK-18 to distinguish NASH from NAFL has since been questioned (287).

Markers of inflammation. Increased circulating concentrations of tumour necrosis factor alpha (TNF- α) have been associated with NASH, insulin resistance, and fibrosis (288-290). The concentrations of serum TNF- α and interleukin 6 (IL-6) were increased in NASH relative to NAFL (291). Additionally, many other cytokines, such as IL-B, macrophage inflammatory proteins, and adipokines, including adiponectin, leptin, resistin, visfatin, and retinol binding protein-4, have been studied as potential biomarkers, but the results have been inconsistent and limited by small sample sizes (292, 293). Use of serum ferritin (SF) has also been examined. In a biopsy cohort of 628 subjects, elevated SF (>1.5-fold increase above upper limit) was associated with NASH, and SF was independently associated with higher NAS (294). SF is, however, elevated in various chronic illnesses and inflammatory states and is thus likely to be a too non-specific marker for NASH. A very recent study on biomarkers of NASH and fibrosis in 648 biopsy-proven patients showed strong association of increased concentrations of activated plasminogen activator inhibitor 1 (aPAI1), a serine protease inhibitor, in subjects with NASH, relative to subjects without NASH or borderline NASH (295).

Novel markers. UPLC-MS based techniques are increasingly replacing many traditional techniques in biomarker research (296). A study in which metabolomic analyses were performed in patients with histologically proven NASH (n=24) or NAFL (n=11) and normal subjects without liver biopsy (n=25) showed increased concentrations of five amino acids (297). The diagnostic performances of these were not compared to routinely available

markers (297). Another study that analysed the lipid and metabolite profile of 467 biopsy proven patients found between 9 and 237 lipids/metabolites to be associated with NASH depending on BMI, suggesting that the degree of obesity influences the relationship between NASH and biomarker concentrations (298). This study also found decreased concentrations of serum PUFA containing TAGs in NASH patients (298). This is in line with the finding of increased absolute overproduction of MUFA and SAFA containing TAGs in the splanchnic area of subjects with NAFLD (299), as well as with a recent study showing absolute and relative excess of these same fatty acids in the circulating lipidome in NASH patients (300).

A proof-of-concept study by Loomba *et al.* (301) of 19 biopsy proven NAFLD patients (NASH n=9, NAFL n=10) vs. 10 non-NAFLD MRI phenotyped controls showed that PUFA metabolites, especially arachidonic acid (AA) -derived eicosanoids differed between NAFL and NASH patients. The best single biomarker to differentiate between NAFL and NASH was 11,12-dihydroxy-eicosatrienoic acid (11, 12-diHETrE) with an AUROC of 1. However, the study was a small pilot study which needs to be validated (301).

4.4 Risk scores

NAFL. Various biomarker-based scores have been developed to diagnose steatosis. The SteatoTest uses a combination of FibroTest-Actitest plus BMI, serum cholesterol, TAG, and glucose adjusted by age and gender (302). The Fatty Liver Index (FLI) includes four variables, i.e. BMI, waist, TAG, and γ GT (303). A simple index of lipid excess includes waist, TAG and gender (304). The Hepatic Steatosis Index contains AST/ALT ratio, BMI, and diabetes (305). To predict the presence of NAFLD, we developed the NAFLD liver fat score using metabolic syndrome, diabetes, insulin, AST, and AST/ALT ratio as the variables, and created a liver fat equation for prediction of the percentage of liver fat (306). Of novel markers, a recent study using UPLC-MS identified a “lipid triplet” to predict NAFLD with a sensitivity and specificity of 69% and 75% respectively. This test performed better than the Liver fat score or the SteatoTest (307).

It has been argued that the scores themselves do not add much to the information provided by clinical, laboratory and imaging studies done routinely on patients with suspected NAFLD. On the other hand, in tertiary care settings where imaging modalities are not always available, the 2016 EASL-EASD-EASO Clinical Practice Guidelines encourage the use of

serum biomarkers and scores, especially NAFLD Liver Fat Score, FLI, or SteatoTest[®] for diagnosis of NAFL.

NASH. As the only reliable method currently available to diagnose NASH is the liver biopsy, there is a dire need for non-invasive methods. At least 23 scores to predict NASH have been developed using routinely available clinical data as well as more specific variables, such as markers of inflammation, oxidative damage, and apoptosis (see Table 2). The NASH Test[®], consisting of alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, γ GT, ALT, AST, TAGs, cholesterol, age, gender, height and weight, developed in France has been externally validated in other French cohorts, but it has a modest AUROC of 0.79 and a very limited sensitivity (308). The U.S. (50) and the European guidelines (47) recommended use of the NAFLD fibrosis score for diagnosis of fibrosis. The NICE guidelines recommended the use of Enhanced Liver Fibrosis[®] score (ELF[®]) for diagnosis of NASH (309). The cost of these tests varies considerably (*e.g.* NAFLD fibrosis score 6 EUR, NASHTest[®] 44 EUR, ELF[®] 120 EUR). A promising novel score combining lipidomics, metabolomics and clinical markers was recently developed (300).

Many of the currently available scores lack external validation, are based on a small sample size, and are not accurate enough to reliably predict NASH (277).

Table 2. Non-invasive scores to predict NASH.

Score	Subjects (n)	Country	Model	AUROC	Se, Sp%	Validation: internal, external	Reference
HAIR	105	Australia	Hypertension, ALT, insulin resistance	0.9 (for ≥ 2 parameters)	80, 89	No, No	(79)
Palekar's score	80	USA	Age, female, AST, BMI, AST/ALT ratio, hyaluronic acid	0.76	74, 66 (for ≥ 2.5 risk factors)	No, No	(310)
NashTest	160 (discovery) 97 (validation) 383 (controls)	France	Age, sex, height, weight, TAG, cholesterol, a2-macroglobulin, apolipoprotein A1, haptoglobin, AST, ALT, GGT, bilirubin	0.79 0.79	39, 92 29, 98	Yes, Yes (in other French cohorts)	(308)
Gholam's model	97	USA	AST, diabetes	0.82	76, 66	No, No	(97)
(no name)	85	Japan	Adiponectin, HOMA-IR, type IV collagen 7S	(NA)	94, 74	No, No	(311)
NASH diagnostics	69 (discovery) 32 (validation)	USA	M30, total CK-18, adiponectin, resistin	0.908 0.70	95, 70 71, 73	Yes, No	(312)

NASH Scoring System in Morbidly Obese	200	USA	Hypertension, diabetes, AST, ALT, sleep apnea, non-black race	0.8	22, 99	Yes, No	(313)
(no name)	101	Argentina	ALT, AST, ALP, γ GT, HOMA-IR, CRP, sICAM-1, BMI, waist	0.79	68, 83	No, No	(314)
OxNASH	73 (discovery) 49 (validation)	USA	Age, BMI, AST, 13-HODE/LA ratio	0.83	81, 97 84, 63	Yes, No	(315)
The Nice mole	310 (discovery) 154 (validation)	France	ALT, CK18, Mets	0.83	84, 86 (NA)	Yes, No	(316)
(no name)	253	USA	Diabetes, ALT, TAG, sleep apnea	0.79	(NA)	No, No	(317)
NASH diagnostic panel	79	USA	Diabetes, gender, BMI, TAG, M30 and M65 (CK18) antigens	0.81	91, 47	No, No	(318)
Apoptosis panel	95 (discovery) 82 (validation)	USA	CK18, sFas	0.93	88, 89 (NA)	Yes, No	(319)
NAFIC score	177 (discovery) 442 (validation)	Japan	Ferritin, INS, type IV collagen	0.85	66, 91 60, 87	Yes, No	(320)

(no name)	66	Brazil	Cholesterol, ALT, AST/ALT, GGT ± US examination	0.73 and 0.82 with US	70, 89	No, No	(321)
Antwerp score	200 (discovery) 113 (validation)	Belgium	ALT, C-peptide, US steatosis scores	0.85	94, 94 42, 36	Yes, No	(322)
'BMI- dependent metabolic model'	374 (discover) 93 (validation)	Spain	BMI dependent metabolic model consisting of various lipids and metabolites	0.87 0.85	71, 92 56, 89	Yes, No	(298)
(no name)	79	Romania	Adiponectin, M65 (CK18), IL-6	0.90	85, 86	No, No	(323)
NASH score	60	Romania	BMI, ALT, AST, ALP, HOMA-IR, M65 (CK18)	0.96	90, 94	No, No	(324)
(no name)	50	Indonesia	AST, TNF-α	0.84	76, 90		(325)
'Clinical model for NASH in diabetic NAFLD patients'	346 (discovery) 100 (validation) 1249 (second validation group; incl. the discovery and validation groups)	USA	White race, BMI, waist, ALT, AST, albumin, HbA1c, HOMA-IR, ferritin	0.82 0.83 0.80	90, 55 91, 46 90, 56	Yes, Yes	(326)

ION score	4458 (US) 152 (biopsy)	USA	In men: Waist-to-hip ratio, TAG, ALT, HOMA In women: TAG, ALT, HOMA	0.88	92, 60	No, No	(327)
NASH ClinLipMet Score	318 (entire cohort) 223 (discovery) 95 (validation)	Finland	glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, and fasting insulin, <i>PNPLA3</i> genotype	0.87 0.88 0.86	86, 72	Yes, No	(300)

5. AIMS OF THE STUDY

Studies I-III were performed to answer the following questions:

- A. Does the genetic variation in *APOC3* contribute to liver fat content and plasma triglyceride and apoC3 concentrations? (I)
- B. What is the prevalence of NASH in Finnish subjects? (II)
- C. How does the *PNPLA3 I148M* gene variant influence circulating TAG concentrations? (III)

6. STUDY SUBJECTS AND STUDY DESIGNS

The subjects for the studies were recruited based on the following criteria, unless otherwise specified: (i) age 18-60 years; (ii) no known illness (acute or chronic) other than obesity or type 2 diabetes based on physical examination, ECG, and standard laboratory tests (blood counts, glucose, thyrotropin, serum creatinine, electrolytes); (iii) alcohol consumption less than 20g per day assessed by interview or questionnaire; (iv) no history of use of toxins or drugs known to induce hepatitis, no clinical signs of hepatitis A, B, or C, autoimmune hepatitis, or evidence of inborn errors of metabolism; (v) no medication that might affect glucose tolerance; and (vi) no pregnancy. Elevated liver enzymes (S-AST, S-ALT, S- γ GT) did not exclude subjects.

All subjects signed a written informed consent. The studies were performed according to the principles of the Declaration of Helsinki and approved by the Ethics Committee of Helsinki University Central Hospital.

Study I.

Objective: To determine whether the genetic variation in *APOC3* contributes to liver fat content and plasma triglyceride and apoC3 concentrations.

Subjects: For the NAFLD study, 417 Finns (310 non-diabetic and 107 type 2 diabetic) were recruited for metabolic studies by contacting occupational health services or by newspaper advertisements. Also, subjects referred to the Department of Gastroenterology because of elevated serum transaminase concentrations using the inclusion criteria as above were recruited. The patients have previously participated in metabolic studies (111, 328-330).

Measurements and study design: ^1H -MRS was used to measure liver fat content. NAFLD was defined as fat accumulation in the liver over 5.6% (55.6mg/g) when measured by ^1H -MRS. Plasma glucose, serum free insulin, fS-HDL, fS-TAG, fS-AST and fS-ALT concentrations were measured. *PNPLA3* (rs738409) and *APOC3* (rs2854116 and rs2854117) were genotyped using the TaqMan PCR method. Plasma apoC3 concentrations were measured enzymatically using ELISA. Metabolic syndrome was defined by using the IDF criteria.

The subjects were genotyped for *APOC3* and divided into wild-type homozygotes (T-455 and C482) and variant allele (T-455C at rs2854116, C-482T at rs2854117) carriers. The same was

done for *PNPLA3*. The genotype groups were compared for clinical and biochemical characteristics, as well as for liver fat content.

Study II.

Objective: To estimate non-invasively the prevalence of NASH

Subjects: Biopsy subjects were recruited in Finland and Italy for discovery and validation of the 'NASH score'. The population-based FIN-D2D cohort was used to estimate the NASH prevalence using the 'NASH score'.

Finnish biopsy cohort. Altogether 296 subjects from Helsinki (n=167) and Kuopio (n=129) eligible for laparoscopic gastric bypass operation were recruited to the study based on the aforementioned inclusion criteria. In addition to the standard laboratory tests outlined above, also serum CK-18 fragments were measured enzymatically using ELISA, and *PNPLA3* was genotyped at rs 739409. Laboratory tests were performed one week before surgery after an overnight fast. Wedge biopsies of the liver were taken at surgery.

Italian biopsy cohort. Altogether 380 subjects of Italian ancestry with a new diagnosis of NAFLD were followed at the Metabolic Liver Diseases outpatient service, Fondazione IRCCS Ca' Granda Ospedale Policlinico. A liver biopsy was obtained from 309 subjects because of persistently abnormal liver enzymes/serum ferritin or a long-lasting history of steatosis with severe metabolic abnormalities. A total of 71 patients were recruited amongst patients eligible for bariatric surgery. Other causes of liver disease were excluded. Alcohol intake among subjects was <30 g/day for men and <20 g/day for women.

FIN-D2D. The study included 2849 patients chosen from a random sample of 4500 subjects aged 45-74 years selected from the National Population Register. The subjects were a part of the implementation project of the national type 2 diabetes prevention programme (FIN-D2D) (331). All subjects gave written consent, and the Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study protocol. All subjects received a self-administered questionnaire on health behaviour and medical history, together with an invitation to clinical examination. Men who consumed ≥ 40 g and women ≥ 20 g of alcohol were excluded from the NAFLD and NASH prevalence estimates. Body measurements were recorded at the site of the study. Glucose tolerance was classified according to WHO 1999 criteria (332).

Laboratory tests (plasma glucose, serum insulin, serum total-, HDL-, and LDL cholesterol, triglyceride, serum ALT, AST and γ GT concentrations) were performed.

Measurements and study design. Clinical characteristics, *PNPLA3* genotype (rs738409) and S-CK18 fragments were measured in 296 biopsy patients to construct (n=195) and validate (n=97) the 'NASH score'. Performance of the scores was analysed using the ROC-curve. The area under the ROC-curve (AUROC) was used to assess the diagnostic performance of the scores. Also, a cut-off for the diagnosis of NASH was defined for a previously validated NAFLD Liver Fat Score (306) (renamed 'NASH Liver Fat Score'). Both scores were validated in 380 Italian biopsy patients. The cut-offs were used in the population-based FIN-D2D study of 2849 subjects aged 45-74 years to estimate the prevalence of NASH.

Study III.

Objective: To examine whether relative concentrations of circulating TAGs between carriers and non-carriers of *PNPLA3 I148M* gene mutation display deficiency of TAGs due to defective lipase activity in the liver.

Subjects: Altogether 372 subjects were recruited by newspaper advertisement, contacting occupational health services or from patients referred to the Department of Gastroenterology due to chronically elevated liver enzymes using the aforementioned inclusion criteria. The study subjects have previously participated in metabolic studies (111, 328-330).

Measurements and study design: A fasting blood sample was taken for measurement of fP-glucose, fS-insulin, fS-LDL cholesterol, total serum cholesterol, fS-HDL cholesterol, fS-TAG, fS-AST, fS-ALT and fS- γ GT, as well as for lipidomic analyses. *PNPLA3* was genotyped at rs739409. Height, weight, waist circumference and blood pressure were recorded. Liver fat content was measured by ^1H -MRS in 75% of subjects and by liver biopsy in 25%. The degree of steatosis in the liver biopsy specimens was converted to liver fat percentage, as measured by ^1H -MRS and liver histology, as previously described (333). NAFLD was defined as in Study I. Lipidomic analyses on serum and plasma samples were performed using Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). Metabolic syndrome was defined as described in Section 7, Methods. The subjects were divided into subgroups based on genotype (*PNPLA3-I148M* mutation carriers and wild-type carriers) and obesity. The groups with a BMI over the median BMI of 33.5 kg/m² were

referred to as ‘obese’, and the rest as ‘non-obese’. To determine whether obesity and the *PNPLA3 I148M* mutation are associated with differences in circulating TAGs in patients with similar liver fat content, we further divided subjects into those with NAFLD due to the *PNPLA I148M* variant (‘PNPLA3 NAFLD’) and to those obese subjects with NAFLD who lack the *I148M* mutation (‘obese NAFLD’).

7. METHODS

7.1 Anthropometric data (studies I, II, III)

Body weight was recorded using a calibrated weighing scale with a 0.1 kg precision with subjects standing barefoot and wearing light indoor clothing. The waist circumference was measured midway between the spina iliaca superior and the margin of the lower rib. Body height was measured to the scale to the nearest 0.5 cm using a ruler attached. Blood pressure was recorded with a random-zero sphygmomanometer (ERKA, Bad Tölz, Germany) after 10-15 minutes of rest with the subject sitting.

7.2 Liver fat content

Histology (studies II, III). A needle liver biopsy was taken under ultrasound guidance in patients with suspected NAFLD or in patients eligible for laparoscopic bypass operation. The liver samples included both core needle and conchotomy material. Liver histology was defined as previously described by Kleiner *et al.* (255). Both, NAFL and NASH subjects were included in the NAFLD group. Liver histology was assessed by two experienced pathologists in the Finnish cohorts in a blinded fashion, first independently and then together to obtain a consensus. In the Italian cohort, one experienced pathologists analysed the liver specimens. Tissue sections were stained with haematoxylin and eosin and saturated with silver for reticulin framework. Trichome stain was used to expose collagen.

¹H-MRS (I, II, III). 1.5T scanners were used (Siemens Magnetom Vision, Erlangen, Germany) to acquire a localized single-voxel proton spectra, as previously described (334). Within the right liver lobe, T1-weighted MRI scans were used for localization of the voxel of interest, that was individually determined for each subject. A short echo time (20 ms) and long repetition time (3000 ms) were used to ensure a fully relaxed water signal. Water signal was used as an internal standard, relative to which the chemical shifts were measured at 4.80ppm. The methylene signal, a representative of intracellular triglyceride, was measured at

1.4ppm. The analysis program VAPRO-MRUI (<http://www.mrui.uab.es/mrui>) was used to quantify the signal intensities. The intracellular triglyceride content (steatosis) was presented as a ratio of the area under the methylene and water peaks multiplied by a 100 to obtain the liver fat percentage, which has been validated against histologically determined lipid content (335). NAFLD was defined as liver fat 55.6 mg triglyceride per gram of liver tissue or 5.56% of liver tissue weight (49). All image data were analysed by physicists who were unaware of the clinical data.

7.3 Definition of metabolic syndrome, diabetes, and obesity (I, II, III)

The Harmonization definition by the International Diabetes Federation was used to define metabolic syndrome (MetS) (332). Diabetes was defined by WHO's criteria (336). Overweight was defined as BMI 25-29 kg/m² and obesity as BMI \geq 30 kg/m².

7.4 Analytical procedures

Plasma glucose concentrations were analysed together with the glucose oxidase method using Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA) (337) (I, II, III). Serum free insulin concentrations were measured using the auto-DELFIA kit (Wallac, Turku, Finland), and C-peptide by radioimmunoassay (338) (I, II, III). FS-triglyceride and fS-HDL cholesterol concentrations were analysed using an autoanalyser with enzymatic kits from Roche Diagnostics (Roche Diagnostics Hitachi, Hitachi Ltd., Tokyo, Japan) (I, II, III). The Friedewald formula was used to determine the LDL cholesterol concentrations (339) (I, II, III). The activity levels of S-AST and S-ALT were analysed as recommended by the European Committee for Clinical Laboratory standards (I, II, III). Plasma ApoC3 concentrations were measured enzymatically by AssayMax human Apolipoprotein C-III enzyme-linked immunoabsorbent assay (ELISA) kit (AssayPro, St. Charles, MO, USA) (I). CK-18 M30 antibody fragments (U/L) were measured using Apoptosense® ELISA (cat. no. 10010) assay (PEVIVA AB, Sweden) (II).

For genotypic data, genomic DNA was extracted from whole blood as previously described (340). TaqMan polymerase chain reaction (PCR) method (Applied Biosystems, Foster City, CA, USA) was used to genotype *PNPLA3* at rs738409 (I, II, III) and *APOC3* at rs2854116 and rs2854117 (I). ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems) was used to carry out post-PCR allelic discrimination (I, II, III).

Lipidomic analyses on citrate plasma (78%) and EDTA plasma and serum (16% and 7%, respectively) samples were carried out using an established platform for ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-MS), based on ACQUITY UPLC (Waters) (341). Studies comparing citrate and EDTA plasma and serum samples from the same subjects did not have statistically significant differences in the lipidomic data. In brief, 10 μ L of serum or plasma sample was diluted with 10 μ L of 0.15 M (0.9%) sodium chloride and 10 μ L of internal standard mixture 1A was added, the mixture containing PC (17:0/0:0), PC (17:0/17:0), PE (17:0/17:0), PG (17:0/17:0) [rac], Cer (d18:1/17:0), PS (17:0/17:0) and PA (17:0/17:0) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) and MAG (17:0/0:0/0:0) [rac], DAG (17:0/17:0/0:0) [rac] and TAG (17:0/17:0/17:0). The lipids are extracted using the mixture of HPLC-grade chloroform and methanol (2:1; 100 μ L). The lower phase (60 μ L) was collected and 10 μ L internal standard mixture 2 was added, containing labelled PC (16:1/0:0-D3), PC (16:1/16:1-D6), and TAG (16:0/16:0/16:0-13C3). ESI+ mode was used to carry out lipid profiling and the data were collected at mass range of m/z 300-1200 with scan duration of 0.2 sec. MZmine 2 software (342) was used for data processing, and the identification of lipids was based on *de novo* identification using tandem mass spectroscopy (MS) or an internal spectral library (341). The data were normalized using one or more internal standards representative of each class of lipids present in the samples.

7.5 Statistical analyses

A *p*-value of less than 0.05 was considered statistically significant in all studies. Calculations were made using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) (I), Stata 9.2 (StataCorp, College Station, TX, USA) (I), SPSS 17.0 (I) or 19.0 (II) for Windows (SPSS, Chicago, IL, USA), or the R 3.0.1 program (II, III).

Study I.

Kolmogorov-Smirnov test was used to test normality. Normally distributed data are reported as means \pm SEM and non-normally distributed data as median (25% percentile, 75% percentile). Non-normally distributed data were analysed after logarithmic (base 10) transformation. Mann-Whitney test was used to compare the differences in liver fat and apoC3 concentration between variant-allele carriers and wild-type homozygotes. Kruskal-Wallis test was used to compare haplotypes. Correlation analyses were carried out using Spearman's non-parametric rank correlation coefficient. To compare differences in binary

variables the Chi-squared test was used. One-way ANOVA was used to compare the genotypic effect on clinical variables and liver fat content. For post-hoc analysis the least significant difference pairwise multiple comparison test was used. Adjusting for use of confounders, such as statins, was carried out using analysis of covariance. The variance explained (%) by *APOC3* and *PNPLA3* genotypes was calculated with multiple linear regression analysis.

Study II.

Altogether 292 bariatric surgery patients with available required data were used to construct and validate a 'NASH' score. This was then used to estimate the prevalence of NASH in a population-based study of 2849 subjects aged 45-74 years.

Development of the 'NASH score'. The biopsy cohort was divided randomly into estimation group (n=195) to develop and validation group (n=97) to validate the model. A second validation group was built using all subjects (n=292). To compare the differences between the estimation and validation groups, the unpaired t-test was used. The odds ratio (OR) and confidence intervals (CIs) for NASH were calculated using the univariate logistic regression analyses. To identify variables that are independently associated with NASH, the variables that were significantly associated with NASH in univariate logistic regression analysis were included in multivariate backward logistic regression analyses. The 'NASH score' was then built using multivariate logistic regression analyses. The performance of the scores was assessed by the receiver operating characteristics (ROC) curve, and the diagnostic accuracy of the scores using area under the ROC-curve (AUROC). The Youden index was used to calculate the optimal cut-off point. The sensitivity (Se), specificity (Sp), negative predictive value (NPV), and positive predictive value (PPV) for the chosen cut-offs were calculated using formulas, as previously described (343). The 95% confidence interval (CI) for the NAFLD and NASH prevalences was calculated using the formula $p \pm 1.96\sqrt{\frac{p(1-p)}{n}}$.

'NASH liver fat score'. For the 'NALFD liver fat score' equation, which had been developed to diagnose NAFLD, a new cut-off to diagnose NASH was calculated using ROC-curves and the Youden index.

Sensitivity analyses. Sensitivity analyses were performed on the new 'NASH score' and the 'NASH liver fat score' by assessing stochastic false-positivity and false-negativity rates in a Bayesian model. To construct the model JAGS and R-package were used on the basis of 2

Markov chains, each containing 5000 “burn-in” samples and 10 000 retained samples to obtain the posterior estimates of the prevalence (344).

Study III

Division of subgroups. The study subjects were divided into groups based on either their genotype (*PNPLA3* variant allele carriers [*PNPLA3*^{I48MM/MI}] vs. homozygotes [*PNPLA3*^{I48II}]) or obesity. The subjects with BMI over the median of 33.4 kg/m² were referred to as the ‘obese’ and those with BMI under the median BMI as ‘non-obese’. Subjects with liver steatosis due to the I148M variant were termed as ‘*PNPLA3* NAFLD’, and obese subjects without the I148M variant as ‘obese NAFLD’.

Analysis of clinical data. Pearson Chi-squared test was used to determine the association of binary variables with *PNPLA3* genotype or obesity. Shapiro-Wilks test was used to test for normality. To compare continuous values between the *PNPLA3* variant and the wild-type and between obese and ‘non-obese’ groups, the Wilcoxon rank-sum test was applied. Medians and 95% CIs were calculated for non-normally distributed, and means and SEM for normally distributed data.

Cluster analysis of lipidomics data. To group lipids with similar profiles in all study samples the Bayesian model-based clustering was applied using an R-package, mclust version 4.0. (345). Log₂-transformation was used on the lipidomics data and each lipid variable was scaled to zero mean and unit variance prior to analysis. For each resulting cluster an average profile was calculated by taking, sample by sample, the mean value of all variables in it. Student's t-test was used to compare the mean values of the cluster profiles between *PNPLA3* variant and wild-type allele groups, and the obese vs. ‘non-obese’ groups.

Analysis of TAG abundances. For all four subgroups mean values and SEMs were calculated for the TAG abundances. After a log₂ transformation, the Student's t-test was used to compare mean values of the TAG abundances between the *PNPLA3* genotype and obese subgroups. To calculate q-values, multiple hypothesis testing was used, as described before (346). To visualize the comparison between the abundances of TAG molecules with respect to their FA chain lengths and the number of double bonds, heatmaps were created. The data values on the heatmaps are a log₂ of the ratio of the mean values of the case group divided by the mean values of the control group. The cells in the heatmap show the significance of the difference in the mean.

8. RESULTS

8.1 Effect of *APOC3* variants on liver fat content (I)

Characteristics of study groups. *APOC3* variant allele (T-455C at rs 2854116, C-482T at rs2854117) and wild-type homozygotes (T-455 and C-482) carriers were comparable with regard to gender, age, BMI, and body weight. No differences were observed in fP-glucose, insulin, HDL, triglyceride, ALT, and AST concentrations. Also, no difference emerged regarding the prevalence of the metabolic syndrome (MetS) or DM2 in the two groups. In the clinical and biochemical characteristics, only the total and LDL cholesterol were slightly higher ($p=0.024$, $p=0.012$) in wild-type than variant allele carriers; other parameters showed no difference (Table 3).

Table 3. Characteristics of *APOC3* wild-type homozygotes (T-455 and C-482) and variant allele (T-455C, C-482T, or both) carriers.

	Wild-type homozygotes	Variant-allele carriers	p-value	p-value adjusted for age and gender
N	53	364		
Age (years)	45±12	44±13	0.58	
Gender (female, % female)	29 (55%)	191 (53%)	0.77	
Metabolic syndrome (%)	68	60	0.30	
Type 2 diabetes (%)	17	27	0.13	
Weight (kg)	91±20	93±23	0.55	0.52
Waist (cm)	106.5±17.5	103.9±17.2	0.21	0.43
BMI (kg/m ²) (% obese*)	32.0±6.7 (59%)	31.5±7.5 (51%)	0.63	0.77
fP-glucose (mmol/l)	6.1±1.9	6.4±2.0	0.30	0.24
fS-insulin (mU/l)	11.6±8.3	10.7±7.4	0.46	0.56
fS-TAG (mmol/l)	1.6±0.8	1.7±1.3	0.77	0.77
fS-HDL cholesterol (mmol/l)	1.4±0.4	1.4±0.4	0.74	0.79
fS-LDL cholesterol (mmol/l)	3.2±1.0	2.8±0.9	0.01	0.01
Total S-cholesterol (mmol/l)	5.3±1.1	4.9±1.0	0.02	0.03
fS-ALT (U/l)	43.6±36.2	43.7±38.7	0.99	0.94
fS-AST (U/l)	32.1±13.8	35.2±22.4	0.17	0.15
Liver fat (%)	4.2 (1.6-12.1)	5.2 (1.4-13.4)	0.53	0.49

NAFLD (%)	43	48	0.56 ^a	0.55
fP-Apolipoprotein C3 (μg/ml)	36.9 (32.5-54.5)	35.8 (28.9-45.2)	0.46	0.46
	(n=19)	(n=113)		

Data are shown as mean ± SD, except for liver fat and apoC3, which are shown as median and 25th and 75th percentiles. ^aChi-squared test. *obesity defined as BMI >30 kg/m².

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Liver fat content. There was no difference in liver fat content (%) between variant allele carriers (5.2% [1.4-13.4]) and wild-type homozygotes (4.2% [1.6-12.1]). The pattern of liver fat distribution (Fig. 2) and the prevalence of NAFLD (48% and 43%, respectively) were similar in the two groups. When the different genotypes at rs2854117 and rs2854116 were analysed separately, the results remained essentially the same. Liver fat content did not differ in subjects with NAFLD between wild-type homozygotes and variant allele carriers (14.0% [9.4-22.9] and 14.5% [9.2-22.9], respectively), and the same was observed for those wild-type homozygotes and variant allele carriers without NAFLD (1.7% [1.0-2.7]) and 1.7% [0.7-3.2]). When grouped according to haplotypes, both liver fat content and plasma apoC3 concentrations were comparable (Table 4).

Figure 2.

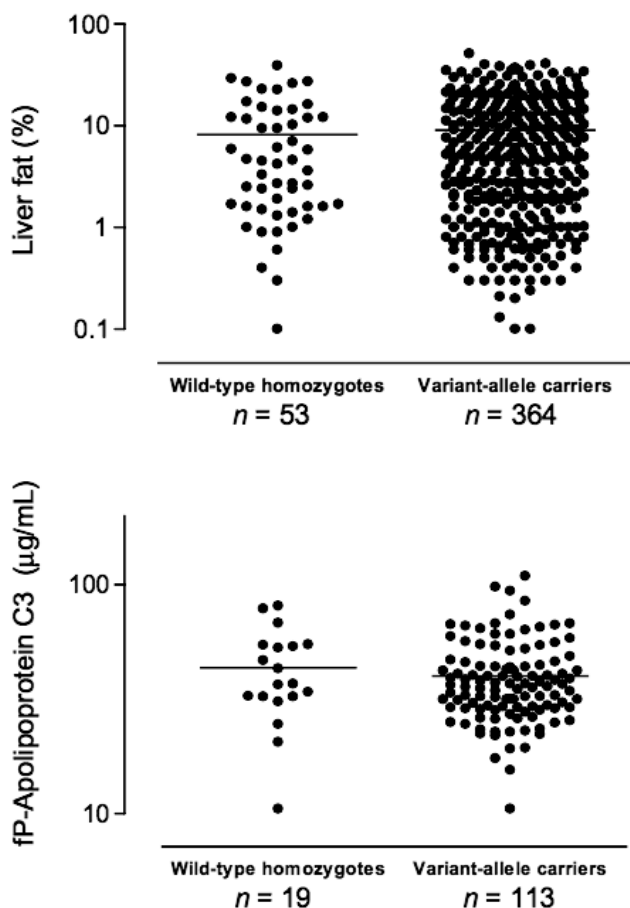


Figure 2. Upper panel compares the liver fat measured with ^1H -MRS and lower panel the plasma apolipoprotein C3 concentrations in *APOC3* wild-type homozygotes (T-455 and C-482) and variant allele carriers (T-455C, C-482T, or both). Reproduced with permission from the copyright holder (347).

Table 4. Liver fat and apoC3 concentrations of the different haplotype carriers of *APOC3* T-455C and C-482T SNPs. Reproduced with permission from the copyright holder (347).

<i>APOC3</i> haplotypes	Number of	Number of	Number of	N	Liver fat (%)	N	fP-apoC3 (µg/ml)
	variant alleles	variant haplotypes	variant loci				
1) C482-T455/ C482-T455	0	0	0	53	4.2 (1.6-12.1)	19	36.9 (32.5-54.5)
2) C482-T455/ C482-C455	1	1	1	38	5.9 (2.7-16.3)	9	32.9 (29.5-42.3)
3) C482-T455/ T482-C455	2	1	2	142	3.5 (1.4-12.2)	33	35.5 (28.1-50.0)
4) C482-C455/ C482-C455	2	2	1	2	2.7	1	40.7
5) C482-C455/ T482-C455	3	2	2	39	10.0 (2.8-19.0)	16	39.8 (36.8-55.7)
6) T482-C455/ T482-C455	4	2	2	127	5.0 (1.3-13.9)	51	34.4 (27.2-44.1)
7) C482-T455/ T482-T455	1	1	1	1	15.1	1	31.7
8) T482-T455/ T482-C455	3	2	2	4	5.9 (3.14-15.9)	2	51.5

Plasma apoC3 concentrations. Plasma from 132 subjects was available for measurement of apoC3 concentrations. There was no difference in the plasma apoC3 concentrations between wild-type homozygotes and variant allele carriers (Table 3, Fig. 3). Also, no difference was seen in apoC3 concentrations between different genotypes at rs2854117 and rs2854116. In variant allele carriers, plasma apoC3 concentrations were positively associated with liver fat ($r=0.33$, $p<0.0017$, Fig. 3), fS-triglycerides ($r=0.39$, $p<0.0001$), total serum cholesterol ($r=0.42$, $p<0.0001$) and LDL cholesterol ($r=0.33$, $p<0.0001$).

Figure 3.

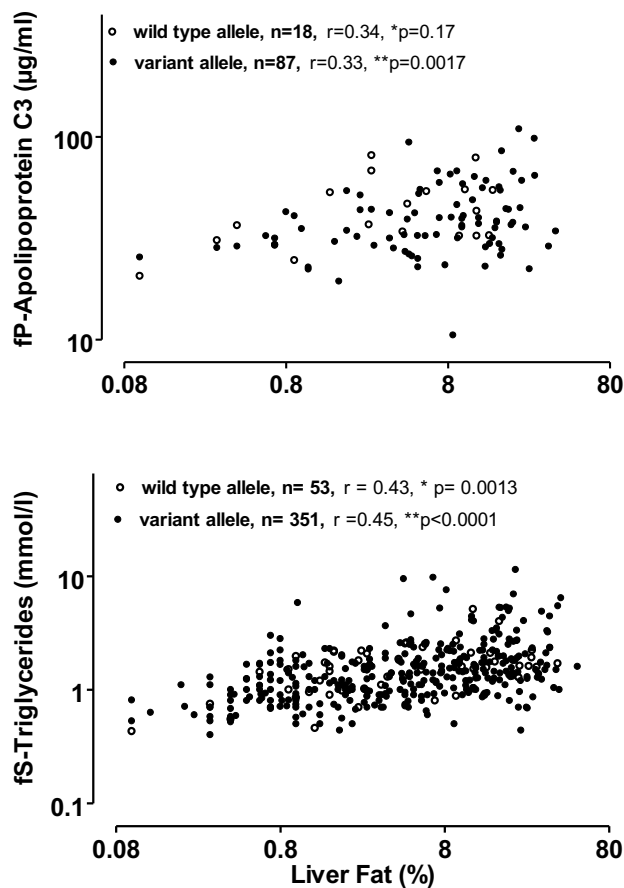


Figure 3. Upper upper panel examines the relationship between liver fat content and plasma apoC3 concentrations and the lower panel between liver fat content and fS-triglycerides in *APOC3* homozygotes (T-455 and C-482) and variant allele carriers (T-455C, C-482T or both). Empty circles represent wild type allele carriers and filled black circles variant allele carriers. Scales have been logarithmically transformed. Reproduced with permission from the copyright holder (347).

Variance in liver fat. Linear regression analysis was used to compare the genotypic effect of *APOC3* and *PNPLA3* variants on liver fat content. *APOC3* alone and *PNPLA3* alone (n=385) accounted for 0% (p=0.33) and 3.2% (p<0.0001), respectively, of the variance of liver fat content. Carriers of the *PNPLA3* (rs738409) GG-genotype had a 2.7-fold (median 11.3%) higher liver fat than carriers of CC-genotype (median 4.2%). Gender, age, BMI, and the SNP at rs738409 in *PNPLA3* predicted liver fat independently in multivariate linear regression analysis.

8.2 Prevalence estimate of NASH (II).

Biopsy studies. In the Finnish biopsy cohort (n=296) 42% had NAFL (n=123), 29% had NASH (n=85), and 5.2% had advanced fibrosis (n=15). In the Italian biopsy cohort (n= 380) 55% had NAFL (n=211) and 45% had NASH (n=169). The clinical characteristics of the two cohorts are shown in Table 5.

Table 5. Clinical characteristics of the patients in the Finnish and Italian biopsy studies.

	Finnish cohort	Italian cohort
N	296	380
Age (years)	47±9	48±12
Gender (% male)	116 (39)	246 (65)
BMI (kg/m ²)	43.7±8.6 (91)	31.7±8.4 (40)
(% obese BMI> 30kg/m ²)		
Type 2 diabetes (n, %)	115 (39)	77 (20)
Metabolic syndrome (n, %)	239 (81)	160 (42)
fS-ALT (IU/l)	52±39	48±33
fS-AST (IU/l)	39±25	23±33
fS-INS (mU/l)	17.0±11.0	20±21
S-ALB (g/l)	37±4	46±3
B-Platelets (x10 ⁹ /l)	264±72	222±62
fS-Triglycerides (mmol/l)	1.6±0.9	1.6±1.4
fS-HDL cholesterol (mmol/l)	1.2±0.4	1.3±0.4
fS-LDL cholesterol (mmol/l)	2.5±0.8	3.2±1.0
<i>PNPLA3</i> (CC/CG/GG) (n, %)	135 (52) / 100 (38) / 26 (10)	152 (40) / 175 (46) / 53 (14)
CK18 (U/L)	473±552	298±204 (n=80)
NAFL (n, %)	123 (42)	211 (55)
NASH (n, %)	85 (29)	169 (45)

Data are shown as mean ± SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, serum albumin; BMI, body mass index; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis. Reproduced with permission from the copyright holder (347)

'*NASH score*'. Variants that were significantly associated with NASH in univariate analyses were examined in multivariate logistic regression analysis. In the discovery group the AUROC was 0.776 (95% CI 0.701-0.852). Backward multivariate regression analyses were used to identify the variables that independently predicted NASH.

This model was used to build the NASH score:

$$-3.05 + 0.562 \times PNPLA3 \text{ genotype (CC=1, CG=2, GG=3)} - 0.0092 \times \text{fS-insulin (mU/L)} + 0.0023 \times \text{AST (IU/L)} + 0.0019 \times (\text{fS-insulin} \times \text{AST}).$$

The optimal cut-off value to predict NASH was determined using the Youden index. In the discovery group, values greater than -1.054 predicted NASH with a sensitivity of 75% and a specificity of 74%.

'NASH liver fat score'. The optimal cut-off to predict NASH for the *'NASH liver fat score'* was 2.122.

Validation of the 'NASH score' and the 'NASH liver fat score' to predict NASH. The *'NASH score'* was first validated in the remaining Finnish biopsy subjects (n=97) and had an AUROC of 0.758 (95% CI 0.626-0.891). NASH was predicted with a sensitivity of 65.2% and specificity of 72.9% for the cut-off value of -1.054.

An external Italian biopsy cohort was used to validate both the *'NASH score'* and the *'NASH liver fat score'*. For the *'NASH score'* the AUROC was 0.759 (95% CI 0.711-0.807). The cut-off of -1.054 predicted NASH with a sensitivity of 39% and specificity of 89%. The AUROC for the *'NASH liver fat score'* was 0.737 (95% CI 0.687-0.787) and predicted NASH with a sensitivity of 93% and specificity of 33% with the cut-off value of -2.122.

Comparison of the ROC curves. In the Finnish biopsy cohort, the *'NASH score'* and the *'NASH liver fat score'* had AUROCs of 0.774 (95% CI 0.704-0.839) and 0.734 (95% CI 0.664-0.805), respectively. The *'NASH score'* had a PPV and NPV of 53% and 86%, and the *'NASH liver fat score'* 54% and 83%, respectively. In the biopsy cohort from Italy the AUROCs for the *'NASH score'* and the *'NASH liver fat score'* were 0.759 (95% CI 0.711-0.807) and 0.737 (95% CI 0.687-0.787), respectively. For the *'NASH score'* the PPV and the NPV were 65% and 74%, and for the *'NASH liver fat score'* 53% and 85%, respectively. Figure 4 compares the ROC curves in the Finnish (left panel) and Italian (right panel) cohorts. The AUROCs of the Finnish and Italian biopsy cohorts did not differ significantly from each other.

Figure 4.

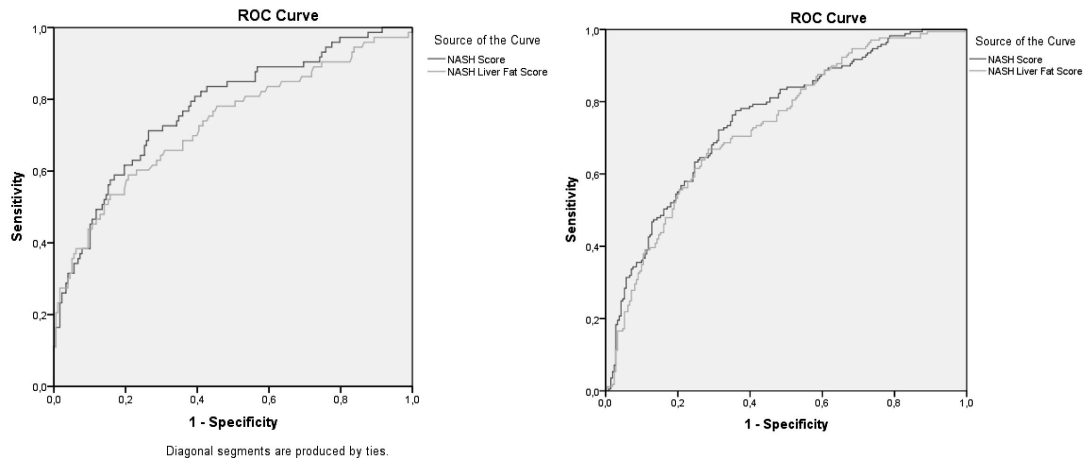


Figure 4. ROC-curves. The left panel represents the ROC curves of the two scores to predict NASH in the Finnish biopsy cohort, and the right panel in the Italian biopsy cohort. The AUROCs were 0.774 (95% CI 0.709-0.839) for the ‘NASH score’ and 0.734 (95% CI 0.664-0.805) for the ‘NASH liver fat score’ in the Finnish cohort, and 0.759 (95%CI 0.711-0.807) and 0.737 (95%CI 0.687-0.787), respectively, in the Italian cohort. The AUROCs did not differ significantly between the cohorts. Reproduced with permission from the copyright holder (348).

FIN-D2D study. Table 6 shows the clinical characteristics of the 2849 subjects, 48% of whom were male. Their mean age was 60 ± 8 years, and the mean BMI was $27.5\pm 4.8\text{kg/m}^2$. 25% were obese, 17% had DM2, and 57% had MetS.

Table 6. Clinical characteristics of the FIN-D2D study subjects.

N	2849
Age (years)	60 ± 8
Gender (% male)	1357 (48%)
BMI (kg/m^2)	27.5 ± 4.8
Type 2 Diabetes (n, %)	479 (17%)

Metabolic Syndrome (n, %)	1625 (57%)
S-ALT (IU/l)	27±17
S-AST(IU/l)	26±13
fS-INS	8.8±16.4
fS-Triglycerides (mmol/l)	1.4±0.8
fS-HDL cholesterol (mmol/l)	1.4±0.3
fS-LDL cholesterol (mmol/l)	3.4±0.9
<i>PNPLA3</i> (CC/CG/GG)	1666 (61.1%) /936 (34.3%) /125 (4.6%)
Score NAFLD* (n, %)	1010 (39.5 % [95%CI: 37.6 - 41.4])
Score NAFL* (score NAFLD– score NASH)	862 (33.8%)
Score-NASH** (n, %)	
NASH Liver Fat Score (cut-off 2.122)	107 (4.2% [95%CI: 3.4 - 5.0])
NASH Score (cut-off -1.054)	149 (6.0 % [95%CI: 5.0 - 6.9])

Data are shown as mean ± SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, serum albumin; BMI, body mass index; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis. *NAFLD calculated based on the NAFLD liver fat score (available for 2554 subjects) (306). **NASH calculated based on the two NASH scores (available for 2497 subjects). Reproduced with permission from the copyright holder (348).

Prevalence estimate. According to the ‘NASH score’, the population-based prevalence estimate of NASH in the D2D cohort was 6.0% (95% CI 5.0-6.9) and according to the ‘NASH liver fat score’ 4.2% (95% CI 3.4-5.0) (Table 6). Based on the ‘NASH score’, in subjects with DM2 in the D2D cohort the prevalence of NASH was 17.6% (95% CI 13.9-21.2), whereas in those without DM2 it was 3.7% (95% CI 2.9-4.5%) (P<0.0001). In the obese the prevalence estimate of NASH was 14.6% (95% CI 11.8-17.4) and in the ‘non-obese’ 3.2% (95% CI 2.4-4.0) (p<0.0001). The prevalence of NASH in men and women was 7.8% and 5.0% (p=0.00528).

Sensitivity analyses. The Bayesian model was used for sensitivity analyses. The sensitivity and specificity of the ‘NASH score’ were assumed to range from 0.7 to 1 based on the results of the Finnish biopsy cohort. Using this method, the population-based prevalence of NASH was 3.60% (95% stimulation limits: 0.16%, 7.69%), Assuming that the sensitivity and specificity of the ‘NASH liver fat score’ ranges from 0.55 to 1 and 0.7 to 1, the prevalence of NASH was found to be 3.1% (95% stimulation limits: 0.2%, 6.8%).

8.3 Triacylglyceride signature in NAFLD (III).

Study group characteristics.

PNPLA3 subgroups. The *PNPLA3*^{148MM/148MI} and the *PNPLA3*^{148II} groups did not differ in age, sex, and BMI (Table 7, Fig. 5). The liver fat content was markedly higher in the *PNPLA3*^{148MM/148MI} group ($10.5 \pm 0.7\%$) than in the *PNPLA3*^{148II} group ($8.6 \pm 0.6\%$, $p < 0.05$). HDL and LDL cholesterol as well as serum insulin were similar in the groups. The *PNPLA3*^{148MM/148MI} group had marginally, but not significantly ($p = 0.10$) lower serum total TAG concentrations than the *PNPLA3*^{148II} group (Table 7).

Obese and ‘non-obese’ subgroups. No difference was present between the obese and the ‘non-obese’ groups concerning age, sex, and the *PNPLA3* genotype (Table 7). In the obese group, the mean BMI was $42.1 \pm 0.5 \text{ kg/m}^2$ and in the ‘non-obese’ group $28.9 \pm 0.3 \text{ kg/m}^2$ (Table 7, Fig. 5). The obese group had a markedly higher liver fat content ($11.65 \pm 0.7\%$) than the ‘non-obese’ group ($7.5 \pm 0.5\%$, $p < 0.0005$) group (Table 7, Fig. 5). Compared with the ‘non-obese’ group, in the obese group the serum insulin concentration ($15.6 \pm 0.7 \text{ mU/l}$ vs. $9.9 \pm 0.5 \text{ mU/l}$, $p < 0.0005$), as well as the TAG concentrations were markedly higher, whereas the HDL and LDL cholesterol concentrations were lower (Table 7).

‘*PNPLA3 NAFLD*’ vs. ‘*Obese NAFLD*’. Forty-seven subjects were found to have ‘*PNPLA3 NAFLD*’ and 51 subjects ‘*obese NAFLD*’. Liver fat was similar between the groups (Table 7). The ‘*PNPLA3 NAFLD*’ subjects had a mean BMI of $29.6 \pm 0.4 \text{ kg/m}^2$ and the ‘*obese NAFLD*’ $41.0 \pm 0.9 \text{ kg/m}^2$ ($p < 0.0001$). The mean serum insulin concentration was markedly higher in the ‘*obese NAFLD*’ group ($15.7 \pm 1.0 \text{ mU/l}$)

than in the ‘PNPLA3 NAFLD’ group $12.3 \pm 0.9 \text{mU/l}$, $p < 0.001$). There were no differences with regard to serum total TAG or HDL and LDL cholesterol between the groups.

Figure 5. Comparison of BMI, liver fat content and fS-insulin between *PNPLA3*^{148MM/148MI} and *PNPLA3*^{148II} groups and between obese and the ‘non-obese’ groups.

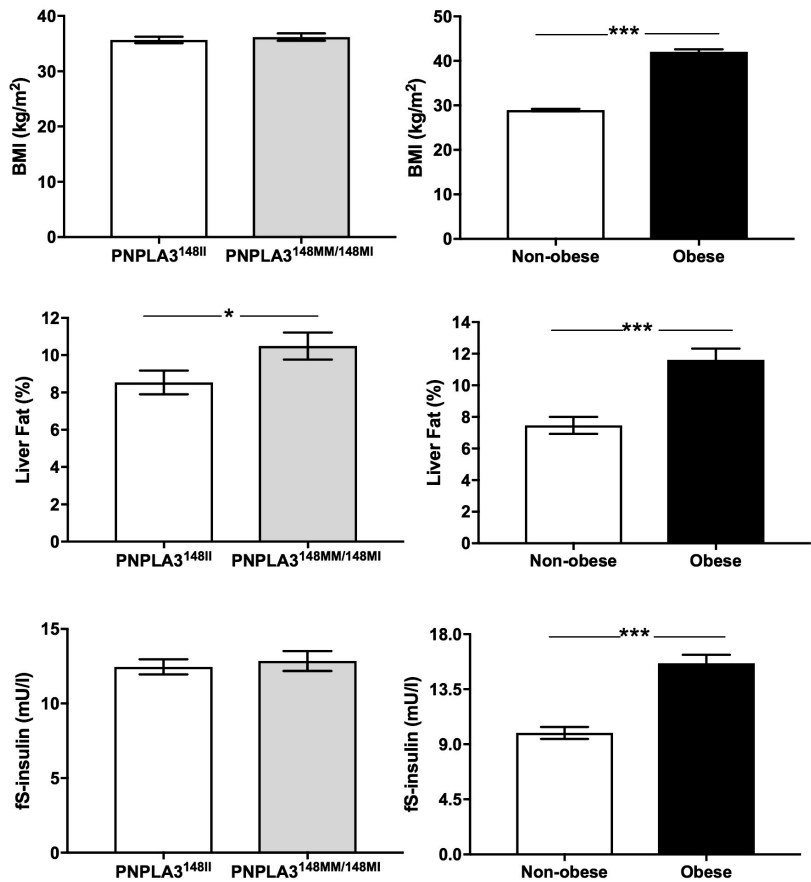


Figure 5. BMI (top panel), liver fat content (middle panel), and fasting serum (fS) insulin concentrations (bottom panel) between *PNPLA3*^{148II} and *PNPLA3*^{148MM/148MI} groups, and between obese and ‘non-obese’ groups. Data are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Reproduced with permission from the copyright holder (348)

Table 7. Clinical characteristics between *PNPLA3*^{148II} and *PNPLA3*^{148MM/148MI} groups and between obese and ‘non-obese’ groups

Variable	<i>PNPLA3</i> ^{148II}	<i>PNPLA3</i> ^{148MM/148MI}	Non-obese	Obese	‘ <i>PNPLA3</i> NAFLD’	‘Obese NAFLD’
n	196	176	<u>165</u>	<u>165</u>	47	<u>51</u>
Age (years)	47 (44, 49)	48 (45, 50)	48 (45, 51)	47 (45, 50)	51 (44, 53)	47 (41, 53)
Gender (% male)	37%	36%	<u>36%</u>	<u>39%</u>	<u>53%</u>	<u>31%*</u>
<i>PNPLA3</i> genotype (GG/CG/CC)	<u>0/0/100 %</u>	<u>14/86/0 %</u>	<u>6/41/53 %</u>	<u>8/41/51 %</u>	<u>17/83/0 %</u>	<u>0/0/100 %</u>
Type 2 Diabetes (%)	25%	34%	<u>26%</u>	<u>36%</u>	36%	33%
Metabolic Syndrome (%)	73%	76%	<u>66%</u>	<u>84%***</u>	83%	<u>82%</u>
BMI (kg/m ²)	<u>35.7±6</u>	<u>36.2±0.7</u>	<u>28.9±0.3</u>	<u>42.1±0.5***</u>	<u>29.6±0.4</u>	<u>41.0±0.9***</u>
Liver Fat (%)	<u>8.6±0.6</u>	<u>10.5±0.7*</u>	<u>7.5±0.5</u>	<u>11.6±0.7***</u>	<u>15.8±1.2</u>	<u>15.9±1.2</u>
fS-insulin (mU/l)	<u>12.5±0.5</u>	<u>12.9±0.7</u>	<u>9.9±0.5</u>	<u>15.6±0.7***</u>	<u>12.3±0.9</u>	<u>15.7±1.0*</u>
fS-ALT (IU/l)	31 (29, 33)	33.5 (29, 36)	<u>29 (26, 33)</u>	<u>33 (30, 37)</u>	46 (34, 64)	33 (29, 44)*

fS-AST (IU/l)	28 (26, 30)	30 (28, 33)	<u>27.5 (26, 30)</u>	<u>30 (28, 32)</u>	34 (30, 45)	31 (27, 35)*
fS-Triglycerides (mmol/l)	1.51 (1.40, 1.70)	1.36 (1.25, 1.50) #	<u>1.4 (1.3, 1.6)</u>	<u>1.5 (1.4, 1.6)</u>	1.60 (1.43, 1.75)	1.60 (1.45, 1.97)
fS-HDL cholesterol (mmol/l)	1.21 (1.16, 1.27)	1.21 (1.17, 1.28)	<u>1.31 (1.24, 1.41)</u>	<u>1.16 (1.11, 1.21)</u> ***	1.16 (1.05, 1.25)	1.17 (1.05, 1.26)
fS-LDL cholesterol (mmol/l)	2.89 (2.70, 3.06)	2.7 (2.54, 2.88)	<u>2.96 (2.85, 3.10)</u>	<u>2.60 (2.32, 2.80)</u> **	3.03 (2.71, 3.30)	2.98 (2.57, 3.28)

Data are shown as median, followed by 95% confidence interval of median or mean \pm SEM. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; #p=0.10, *p<0.05, **p<0.005, ***p<0.0005. †*PNPLA3* genotype (%) at rs 738409 where C is the common allele and G the variant allele (1148M). Reproduced with permission from the copyright holder (348).

Cluster analysis.

Altogether 413 molecular lipids were measured using the UPLC-MS based analytical platform, and 161 of these were identified. The lipidome was first assessed by clustering the data into a cluster subset of 11 lipid clusters using Bayesian model-based clustering (345). To a large extent, the clusters followed different functional or structural groups of lipids. Only LC1, which contained only TAGs, differed markedly between the *PNPLA3*^{148MI} and *PNPLA3*^{148II} groups (Fig. 6). Therefore, this cluster was the target for further analysis. There were also differences in the lipid clusters between the obese and the ‘non-obese’ groups, but they were not included in further analysis.

Figure 6.

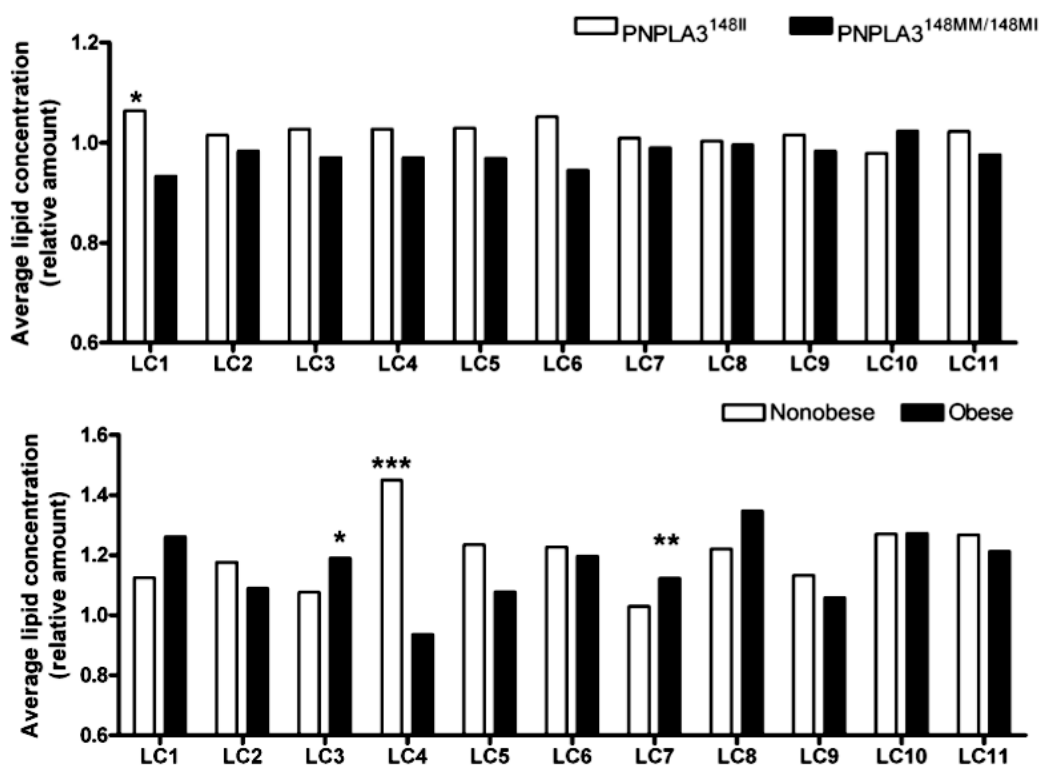


Figure 6. The upper panel represents the mean lipid concentrations within each cluster between *PNPLA3*^{148II} and *PNPLA3*^{148MM/148MI} groups and the lower panel between obese and ‘non-obese’ groups. Statistical comparison performed by two-sided *t* test. **p*<0.05, ***p*<0.01, ****p*<0.001. Reproduced with permission from the copyright holder (349).

Absolute TAG concentrations. In the $PNPLA3^{148MM/148MI}$ group the absolute concentrations of several TAG species were significantly lower than in the $PNPLA3^{148II}$ group. These TAGs consisted of both major (most abundant) and minor TAG species, most of them containing an oleic acid (18:1) acyl group.

Compared with the ‘non-obese’ group the absolute concentrations of several TAGs were markedly increased in the obese group. The most abundant TAG was (16:0/18:1/18:1), which was higher in the obese group (168 ± 7 mmol/l) than in the ‘non-obese’ group (148 ± 5 mmol/l, $p=0.0042$). In contrast, the same TAG (16:0/18:1/18:1) was lower in the $PNPLA3^{148II}$ group (148 ± 4 mmol/l) than in the $PNPLA3^{148MM/148MI}$ group (169 ± 6 mmol/l, $p=0.026$). Also, the absolute concentrations of various other, long-chain fatty acyl-containing TAGs (with 51-58 carbon bonds and 2-9 double bonds) were markedly higher in the obese group. In contrast, the obese group had lower absolute concentrations of short-chain TAGs (42-22 carbon bonds) than in the ‘non-obese’ group (Fig. 8, left panel). A similar pattern was observed in absolute concentrations of TAGs between the ‘obese NAFLD’ vs. the ‘PNPLA3 NAFLD’ groups (Fig. 9). The comparison of absolute concentrations of TAGs between the $PNPLA3$, the obese vs. ‘non-obese’, and the NAFLD subgroups are shown as heatmaps in Figures 7-9 (left panels).

Figure 7. TAG concentrations in the *PNPLA3* subgroups.

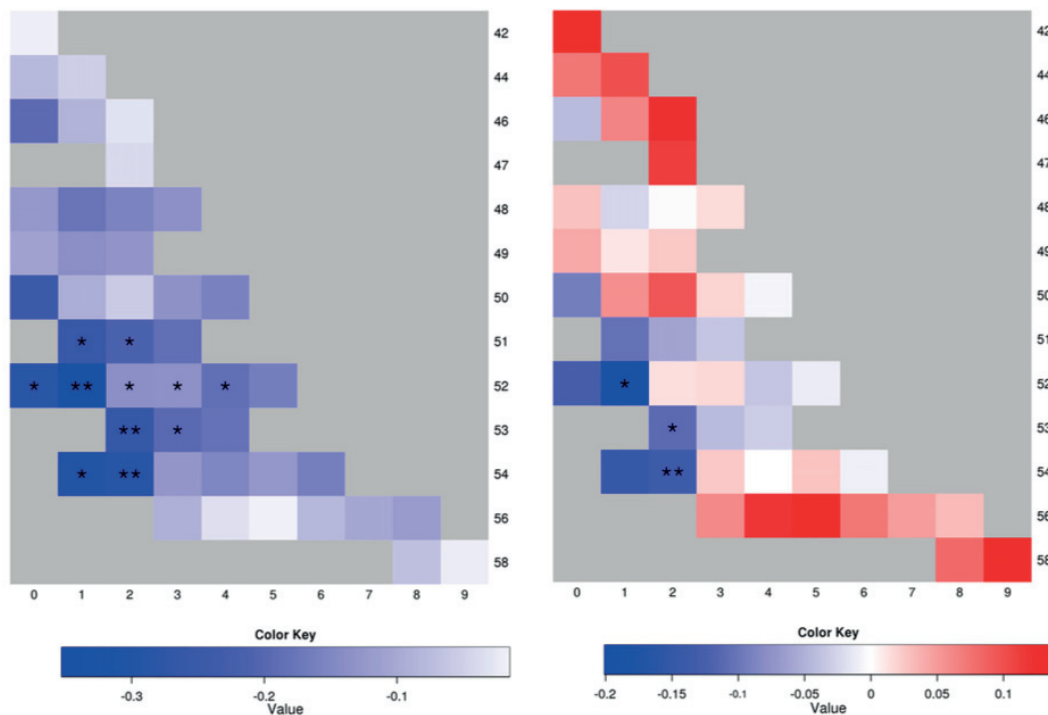


Figure 7. The left panel represents the absolute and the right panel the relative concentrations of TAGs between *PNPLA3*^{148MM/148MI} and *PNPLA3*^{148II} groups. The colour code indicates the logarithm of the ratio between means of the groups for an individual TAG. Blue indicates a decrease in the variant allele carriers (*PNPLA3*^{148MM/148M}) relative to non-variant allele carriers (*PNPLA3*^{148II}). Reproduced with permission from the copyright holder (349).

Figure 8. TAG concentrations in the obese and the ‘non-obese’ subgroups

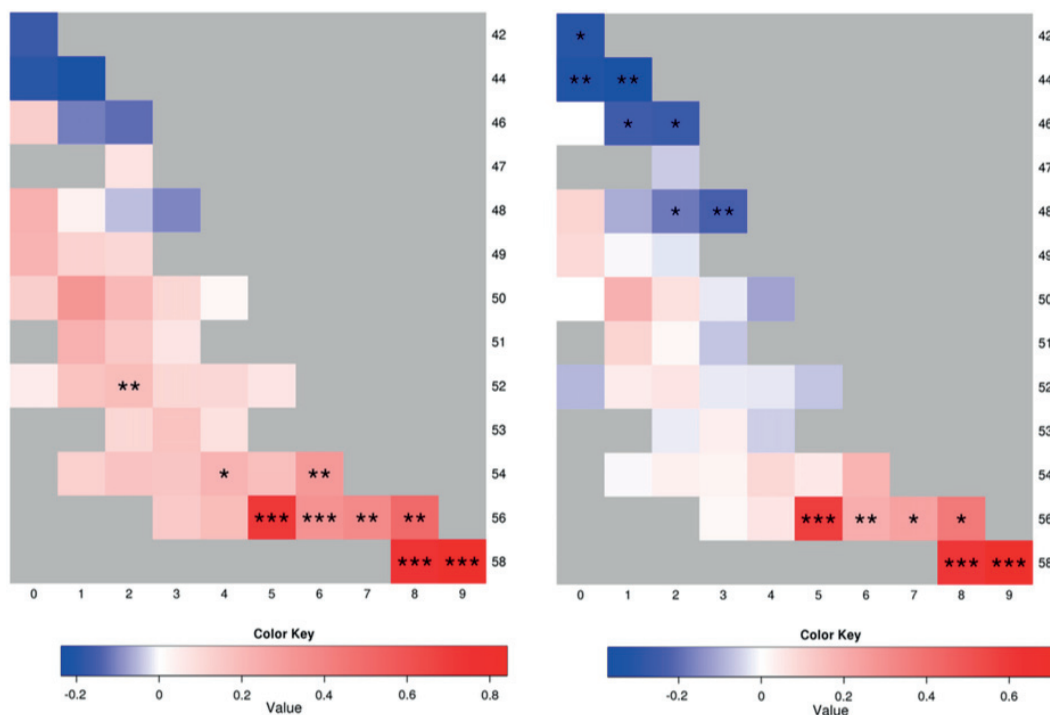


Figure 8. The left panel represents the absolute and the right panel the relative concentrations of TAGs between Obese and ‘Non-obese’ groups. The colour code indicates the logarithm of the ratio between means of the groups for an individual TAG. Blue denotes a decrease and red an increase in the obese relative to the ‘non-obese’. Reproduced with permission from the copyright holder (349).

Relative TAG concentrations. The relative TAG distribution was calculated as concentration of individual TAGs divided by all measured TAGs by UPLC-MS. In the *PNPLA3*^{148MM/148MI} group, the relative concentrations of three long-chain TAGs were markedly decreased as compared with the *PNPLA3*^{148II} group: 52:1 (TAG [16:0/18:0/18:1]), 53:2 (TAG [17:0/18:1/18:1]), and 54:2 (TAG [18:0/18:1/18:1] or TAG [16:0/18:1/20:1]) (Fig. 7, right panel). This pattern of distribution differed markedly from that seen in the obese vs. the ‘non-obese’ groups (Fig. 8, right panel). In the obese group, long-chain polyunsaturated TAGs were relatively increased, whereas the relative concentrations of short-chain TAGs with a few double bonds were decreased (Fig. 9). The differences observed in the absolute

concentrations of 51-54 carbon chain TAGs between the obese and the ‘non-obese’ groups disappeared when analysed using their relative concentrations (Fig. 8). The relative distribution pattern of TAGs between the ‘PNPLA3 NAFLD’ and the ‘obese NAFLD’ groups showed differences in the most abundant TAGs 52:2 and 52:3, but otherwise approximated the pattern seen between the obese and the ‘non-obese’ groups (Fig. 8).

Figure 9. TAG concentrations in the ‘PNPLA3 NAFLD’ and ‘obese NAFLD’ groups

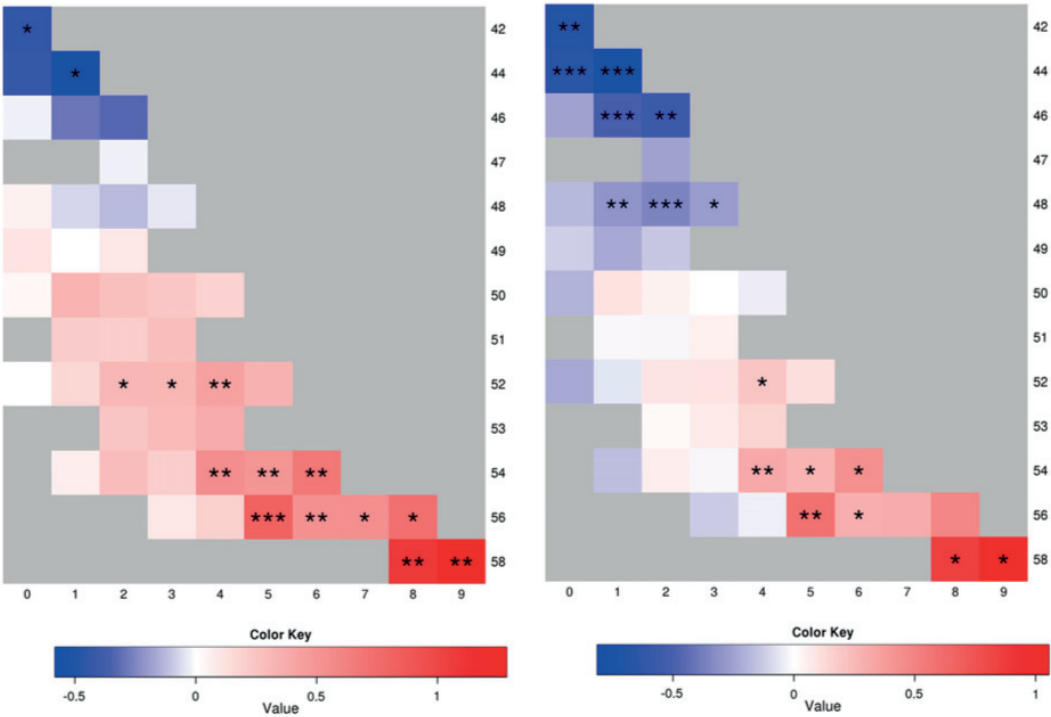


Figure 9. The panel on the left represents the absolute and the panel on the right the relative concentrations of TAGs between ‘PNPLA3 NAFLD’ and ‘obese NAFLD’ groups. The colour code indicates the logarithm of the ratio between means of the groups for an individual TAG. Blue denotes a decrease and red an increase in the ‘PNPLA3 NAFLD’ groups compared with the ‘obese NAFLD’ group. Reproduced with permission from the copyright holder (349).

9. DISCUSSION

The global obesity epidemic parallels the increases in metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), but not all obese subjects develop these conditions, and lean individuals can be insulin-resistant and/or have NAFLD (350, 351). Family studies suggest the heritability of NAFLD to be considerable (141). In the present study, we examined heterogeneity of NAFLD by investigating whether genetic variants in *APOCIII* are associated with NAFLD (I) and whether *PNPLA3 I148M* gene variant influences the circulating lipidome in a different way than NAFLD associated with obesity and insulin resistance (III). Only a fraction of patients with simple steatosis progresses to develop NASH. No data exist on the prevalence of NASH in population-based studies using a biopsy-validated score for NASH. Therefore, we developed a clinical score to predict NASH and used it to estimate the population-based prevalence of NASH (II).

9.1 Effect of APOC3 variants on liver fat content (I)

In Study I variants in *APOC3* alone explained 0% ($p=0.33$), *PNPLA3* alone 3.2% ($p<0.0001$), and the two combined 2.9% ($p=0.18$ for *APOC3* and $p<0.0001$ for *PNPLA3*) of the variance in liver fat. The *APOC3* wild-type homozygotes (T-455 and C-482) and variant allele carriers (T-455C at rs2854116, C-482T at rs2854117, or both) did not significantly differ with respect to liver fat content, NAFLD prevalence, plasma TAG or apoC3 concentrations. These data suggest that in the present study group *APOC3* gene variants do not confer susceptibility to increased liver fat content.

A study by Petersen *et al.* (10) in a cohort of 95 Asian Indian men and a validation cohort of 164 non-Asian Indian men had opposing results to ours. In their study, the prevalence of NAFLD was higher in the variant allele carriers, who also had higher TAG and apoC3 concentrations than the wild-type carriers (10). As in our study, the liver fat content was measured using H-MRS. Our study cohort differed from that of Petersen *et al.*, as our subjects were obese and had metabolic abnormalities, whereas those in the study of Petersen *et al.* had a normal BMI and lower prevalence of NAFLD. On the other hand, the advantage of our study is a larger sample size (417 vs. 95 and 164 subjects). Our study could not replicate the association between *APOC3* variants and liver fat observed by Petersen *et al.* (10). Thus, ethnicity may have had an influence on the association between *APOC3* genetic variation and liver steatosis. Nonetheless, consecutive studies in Hispanic, European

American, African American and European subjects have also failed to confirm the association between *APOC3* variants and NAFLD (352-356).

The robust association between *PNPLA3* and NAFLD has been confirmed in seven of eight genome-wide association studies (122). In the study of Petersen *et al.* (10), *APOC3* variants alone explained 11.0% and *PNPLA3* alone 6.5% of the variance in risk of NAFLD. Our study did not find an association between *APOC3* and liver fat content, whereas the *PNPLA3* I148M explained 3.2% of the variance in liver fat content. The larger cohort or the wider distribution of liver fat in our study could explain the lower percentage.

The differences observed in the variance in liver fat could also be explained by differences in prevalence of the GG genotype at rs738409, which was 6.6% in our study cohort, being comparable to the reported frequencies in other European cohorts (202, 357). This data was not reported by Petersen *et al.* (10). One limitation in our study is that one of the SNPs, the rs2854116, deviated from the Hardy-Weinberg equilibrium ($p=0.03$), whereas the other genotype frequencies at rs738409 and rs2854117 were in equilibrium.

Consistent with previous studies, the plasma apoC3 concentrations were positively correlated with plasma TAG and LDL concentrations (358-361). In contrast to previous studies (10, 362) we did not observe hypertriglyceridemia in the *APOC3* variant allele carriers in our cohort, which could be due to limited availability of apoC3 data ($n=132$ subjects). ApoC3 is known to inhibit LPL (363), and thus, its higher availability could lower the clearance of plasma TAGs, reflected as a positive correlation between the two. On the other hand, as most of the circulating apoC3 is found to be bound to VLDL, the positive correlation could be explained by the increased VLDL-TAG production seen in NAFLD (363). As we lack the turnover data for apoC3 and VLDL, we cannot differentiate between these two possibilities.

In conclusion, variants in *APOC3* do not appear to explain variance in liver fat content in Finns and, unlike in *PNPLA3*, ethnicity could play a role in the effect of genetic variation in *APOC3* on NAFLD.

9.2 'NASH score' and population-based prevalence estimate of NASH (II)

In Study II, to our knowledge we established for the first time, a histologically validated score to predict NASH and to estimate the population-based prevalence of NASH. In the Finnish cohort of 296 bariatric surgery patients, AST, the *PNPLA3* genotype, and fasting

insulin were found to best predict NASH. These were used to create the 'NASH score', which had an AUROC of 0.774 (95% CI 0.709-0.839) in the entire Finnish cohort. The score was validated in an Italian cohort consisting mainly of non-bariatric surgery patients with a mean BMI approximating that of the D2D cohort. The AUROC of the 'NASH score' in the Italian cohort was 0.759 (95% CI 0.711-0.807), being comparable to that in the Finns. We also determined new cut-offs for NASH for a previously validated NAFLD liver fat score (306). This score, termed the 'NASH liver fat score', performed more modestly than the 'NASH score', but the AUROCs were again similar in the Finnish (0.734 [95% CI 0.664-0.805]) and the Italian cohorts (0.737 [95% CI 0.687-0.787]). The NASH prevalence was estimated in the population-based D2D cohort to be 6.0% using the 'NASH score' and 4.2% using the 'NASH liver fat score'. The mean prevalence estimates for the scores using the Bayesian sensitivity analyses were 3.6% and 3.1%, respectively.

The most important limitation in our study is the modest performance of the 'NASH score'; the AUROC is good at best. The AUROC is similar to that seen in a meta-analysis of 494 severely obese French subjects (364), but is somewhat worse than the NICE model developed in a cohort of 464 morbidly obese patients, which consisted of the parameters ALT, CK18, and metabolic syndrome (316). The 'NASH liver fat score' had a moderate AUROC of 0.73.

Additional scores have been developed after the Study II, including a clinical model for NASH in diabetic NAFLD patients, containing the parameters of white race, BMI, waist, ALT, AST, albumin, HbA1c, HOMA-IR, and ferritin (326). This score had a slightly better AUROC than that of the 'NASH score' but was developed to diagnose NASH only in diabetics. The NASH ClinLipMet Score was developed in a Finnish biopsy cohort of 318 subjects and had a markedly better AUROC (0.87) than the 'NASH score' (300). As a drawback, in addition to clinical data, the score contains variables of lipids and metabolites, which are not yet routinely available in the clinic (300).

The prevalence estimate of NASH based on the 'NASH score' and 'NASH liver fat score' is likely to be a gross overestimation of the true prevalence of NASH. This is supported by the lower prevalence estimate for both scores when using the Bayesian model. When applying the 'NASH score', a sensitivity of 71.6% means that 28.4% of those with NASH will be missed, whereas a specificity of 73.5% means 26.5% will be wrongly diagnosed as having the disease. The number of patients with a false-positive diagnosis is therefore too high for

the ‘NASH score’ to be useful in the clinics for diagnosis of NASH. The Bayesian estimate of the mean prevalence rate and its confidence limits further highlights the limitations of the score. Nonetheless, the ‘NASH score’ could be of use in identifying patients needing referral to a hepatologist.

The NASH prevalence of 29% in the Finnish biopsy cohort was comparable to previous studies, in which the prevalence has ranged between 18-37% (77-79, 96, 97, 317, 365-368). Although the ‘NASH score’ was developed in a morbidly obese cohort and applied to a less obese D2D group, we are unaware of data showing that NASH would differ in less obese subjects. To overcome this limitation, both the ‘NASH score’ and the ‘NASH liver fat score’ were validated in the Italian cohort, which has a BMI was similar to that in the D2D cohort.

Another population-based estimate of NASH was carried out in the NHANES survey by Younossi *et al.* (369). In that study, NASH was assumed in patients who had NAFLD in combination with diabetes or HOMA > 3 (369), *i.e.* a clinical evaluation rather than a histologically validated score. Using this definition, the prevalence estimate of NASH in our biopsy cohort was markedly higher than the true prevalence of NASH (44% vs. 29%), suggesting that the NHANES survey prevalence estimate is an overestimation of the true prevalence of NASH (369). The prevalence of 1.1% in the 2005-2008 NHANES survey may seem low compared with the prevalence estimate of ~5% in the D2D sample, but the study samples differ considerably in their mean age, for example, which was ~45 years in the NHANES study (369) and 45-74 years in the D2D cohort. A systematic review from 2011 estimated the prevalence of NASH to be 3-5% (58).

NASH prevalence is dependent on ethnicity (58). As the NAFLD liver fat score has been validated in the diagnosis of NAFLD in other Caucasian subjects, such as Italians (370) and the Dutch (371), the present prevalence estimate of NASH could be applicable to Caucasians. In conclusion, Study II aimed to create a NASH score after testing for major factors previously found to be associated with NASH (50), including fS-insulin, *PNPLA3* genotype, CK-18 fragments, and such clinical parameters as age, gender, liver enzymes, albumin, platelets, glucose, BMI, and waist circumference. A NASH score was developed and validated, and subsequently used to estimate the population prevalence of NASH, which was found to be ~5%.

9.3 Triacylglyceride signature and subtypes in NAFLD (III)

In Study III, we found the circulating TAG profiles to be markedly different between NAFLD associated with the *PNPLA3* I148M variant independent of obesity, and NAFLD associated with obesity independent of the *PNPLA3* genotype. Both the ‘PNPLA3 NAFLD’ and the ‘obese NAFLD’ showed specific changes in the absolute and relative distribution of circulating TAGs, which can be associated with obesity/insulin resistance rather than with liver total TAG concentrations (Fig. 9). The relative TAG distribution was analysed to eliminate the effect of differences in total TAG concentrations.

The 372 subjects were divided into two groups on the basis of their *PNPLA3* genotype at rs739409. One group contained one (GC) or two (GG) of the variant alleles (*PNPLA3*^{148MM/MI}), and the other group was homozygous for the wild-type allele (CC) (*PNPLA3*^{148II}). Unsurprisingly, the group containing the variant allele had markedly higher liver fat content than the wild-type homozygotes, and the *PNPLA3*^{148MM/MI} subjects did not exhibit hypertriglyceridemia or low HDL cholesterol concentrations. This is in agreement with the majority of previous studies showing that fatty liver due to the I148M mutation in *PNPLA3* is not accompanied by the metabolic changes usually seen in NAFLD (3, 6). Serum total triglycerides were somewhat lower in the *PNPLA3*^{148MM/MI} group than in the *PNPLA3*^{148II} group, which is also in line with other studies (204, 372, 373).

The increased liver fat in the *PNPLA3*^{148MM/MI} group was not associated with fasting insulin concentrations (Fig. 5). Hepatic insulin sensitivity can be assessed using fasting insulin, although the direct method is preferred (329). Normal fasting insulin concentrations in subjects with ‘PNPLA3 NAFLD’ are in line with earlier studies reporting fasting insulin concentrations (155, 204, 374). Included in these studies is our previous study on insulin sensitivity of the liver in 109 subjects measured directly using the euglycemic-hyperinsulinemic clamp technique (5), as well as another study performing direct measurement of insulin sensitivity (8). At least two studies have shown opposing results. These include the study on morbidly obese subjects by Palmer *et al.* (373), as well as a study in the Chinese, both reporting an association between the *PNPLA3*^{148MM/MI} variant and hyperinsulinemia (375). In the latter study, however, carriers of the *PNPLA3* mutation were more obese than the wild-type carriers. To sum up, the majority of studies propose the ‘PNPLA3 NAFLD’ to not be accompanied by insulin resistance.

When comparing the lipid clusters (LCs) between the *PNPLA3*^{148MM/MI} and *PNPLA3*^{148II} groups, only one LC containing only TAGs (Fig. 6) was found to differ, and thus, was included in further analysis. When comparing the *PNPLA3* groups, the relative distribution pattern of individual circulating TAGs showed that TAG substrates, especially those that contained an oleic acid (18:1) moiety preferred by adiponutrin in the liver of mice overexpressing *PNPLA3*^{148MM/MI} (164), were markedly depleted in the variant allele carriers (Fig. 7). The differences were apparent in relatively minor TAG species, although a tendency for relative depletion in the 'PNPLA3 NAFLD' group was also seen in the more abundant TAG species containing the 18:1 FAs. Therefore, 'PNPLA3 NAFLD' might not be characterized by an increase in VLDL production, as is the case in 'obese NAFLD' (166). It has been shown in humans that, irrespective of the extent of steatosis, *PNPLA3* variant allele carriers have a lower rate of VLDL₁ and apoB100 production rather than clearance, which is in line with our finding (166). Also, the insulin sensitivity of lipolysis is comparable between the variant allele carriers and the non-carriers (174). Therefore, depletion instead of an increase of a subset of circulating TAGs seen in our study supports the hypothesis of the *PNPLA3* I148M variant allele preventing (169) rather than stimulating (168) hepatocellular TAG synthesis.

It remains unclear how the I148M variant allele impedes the coupling of stored TAGs and VLDL synthesis. As most TAGs which are stored as lipid droplets undergo hydrolysis before being reassembled into VLDL-TAG (376), the mutation in *PNPLA3* could prevent TAG lipolysis at the surface of lipid droplets via manipulation of the adipose triglyceride lipase (ATGL) activity (377), which could be reflected as decreased VLDL production. In line with this, *in vitro* data with cells overexpressing the *PNPLA3* I148M variant allele show a higher neutral lipid content and a decreased secretion of apoB100 relative to the wild-type carriers (166). In addition, theoretically, the decreased TAG lipolysis could lead to the accumulation of metabolically inert TAGs in lipid droplets, thus reducing the amount of harmful lipid intermediates such as diacylglycerides and ceramides (376). This would explain the lack of insulin resistance in the variant allele carriers. Consistent with this hypothesis, cells overexpressing the *PNPLA3* variant have larger lipid droplets (377), and similar increases in liver fat are associated with a ceramide-enriched liver lipidome in 'obese NAFLD' but not in 'PNPLA3 NAFLD' (11).

Dividing the 372 subjects into obese and ‘non-obese’ groups based on their median BMI, showed the liver fat content to be markedly higher in the obese group (Table 7). The obese group displayed various alterations in the LCs (Fig. 6) and in the relative TAG distribution (Fig. 8) compared with the ‘non-obese’ group. These differences could be due to obesity/insulin resistance or the difference in liver fat content. Comparing the ‘obese NAFLD’ and ‘PNPLA3 NAFLD’ groups, similar differences in the relative TAG profiles were observed (Fig. 9) as in the ‘obese’ and ‘non-obese’ groups. This implies that the differences in the TAG profiles were not attributable to higher liver fat content *per se*. The lack of specific TAGs in the *PNPLA3*^{148MM/MI} group relative to the *PNPLA3*^{148II} group was not the case when comparing the ‘PNPLA3 NAFLD’ and ‘obese NAFLD’ groups. This implies that the influence of obesity/insulin resistance on the TAG profile is more important than the relatively minor changes seen in the ‘PNPLA3 NAFLD’. The differences observed between the obese and the ‘non-obese’ TAG profiles could be explained by either clearance of VLDL or its production (378). As the inability of insulin to normally suppress VLDL synthesis appears to be the most important mechanism of hypertriglyceridemia in obese NAFLD subjects (379), in this case increased VLDL production seems more likely. It has been shown that the VLDL composition in NAFLD patients resembles the composition of hepatocellular TAGs, implying that ‘obese NAFLD’ subjects’ liver overproduces VLDL-TAGs proportionally to increased TAG production (24).

The lipid and fatty acid compositions of lipoprotein fractions have been previously measured in subjects with a wide range of insulin sensitivity (299), as has the rate of production of individual TAGs in the splanchnic bed of NAFLD patients (299). Insulin resistance was positively correlated with the relative serum concentrations of 16:0, 16:1 and 18:0 esterified fatty acids, whereas the concentrations of essential fatty acids were negatively correlated with insulin resistance (380). Also, liver biopsy samples from fatty livers are enriched in TAGs containing saturated and monounsaturated fatty acids (381). In line with this data, the absolute serum concentrations of the abundant, saturated and monounsaturated fatty acid containing TAGs were markedly increased in the obese group compared with the ‘non-obese’ group. Nonetheless, when examining the relative concentrations of these TAGs they did not appear to differ between the obese and the ‘non-obese’ groups. Instead, the minor, polyunsaturated TAG species were relatively upregulated, and the short-chain TAG species with less double bonds were down-regulated in the obese group (Figs. 8 and 9). The reason behind these changes cannot be currently established.

In conclusion, our data suggest that NAFLD is heterogeneous, and at least two different forms exist. The ‘PNPLA3 NAFLD’ is marked by absolute and relative deficiencies in specific circulating TAGs, supporting the hypothesis of the *PNPLA3* variant allele being a loss-of-function mutation impairing lipolysis, rather than a gain-of-function mutation. When comparing ‘obese NAFLD’ and ‘PNPLA3 NAFLD’, various changes in the absolute and relative TAG concentrations are seen, which are attributable to obesity/insulin resistance rather than to increased liver fat *per se*.

10. SUMMARY AND CONCLUSIONS

I) Genetic variants in *APOC3* do not appear to contribute to liver fat in Finns. Therefore, unlike variants in *PNPLA3*, ethnicity might influence the effect of *APOC3* on NAFLD.

II) We developed and validated a score to predict NASH, which included the parameters AST, *PNPLA3* genotype, and fS-insulin. This ‘NASH score’ was used to estimate the prevalence of NASH in a population-based D2D study. The prevalence of NASH was ~5%.

III) The aetiology of NAFLD influences circulating TAG concentrations. ‘*PNPLA3* NAFLD’ is characterized by absolute deficiency of circulating TAG concentrations, supporting the loss-of-function theory in the *PNPLA3* I148M variant. ‘Obese NAFLD’ is associated with absolute increases in circulating TAGs. These changes can be attributed to obesity/IR rather than to increased liver fat *per se*.

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Helsinki, October 2017

A handwritten signature in black ink, reading 'Jenni Hyysalo'. The signature is fluid and cursive, with the first name 'Jenni' written in a large, flowing script and the last name 'Hyysalo' in a slightly more compact but still cursive style.

Jenni Hyysalo

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